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TUMOUR MICROENVIRONMENT IN SEROUS OVARIAN CANCER

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To my big messy family,

to my solid roots

and to myself

"…unless it comes out of
your soul like a rocket,
unless being still would
drive you to madness or
suicide or murder,
don't do it.
Unless the sun inside you is
burning your gut,
don't do it."

So you want to be a writer
Charles Bukowski

ABSTRACT

Ovarian cancer is the seventh most common malignancy in women worldwide and the most lethal gynaecological malignancy in developed countries. The epithelial subtype is divided in five main histologic groups, of which the high-grade serous is the most common subtype. Advanced stage at diagnosis, poor prognosis and high incidence of resistance to therapy constitute the most important challenges for patients with ovarian cancer. The search to identify new prognostic and predictive markers represents one of the major goals in the research field of high-grade serous ovarian cancer (HGSOC).

During tumour development and progression, the ovarian stroma, constituted by blood vessels, fibroblasts, smooth muscle cells and connective tissue, sustains cancer cells through synergistic paracrine communications. This thesis aimed at studying the components of tumour microenvironment in serous ovarian cancer, especially HGSOC, and their possible association with survival and response to therapy.

We identified PDGFRβ positive stroma fibroblasts and perivascular cells as a determinant of poor survival in serous ovarian cancer. When we characterized PDGFRβ positive stroma and vasculature of ovarian cancer, compared to renal and colorectal cancer, we found both similarities and differences. We also studied the interaction between cancer-associated fibroblasts and immune cells and noted an inhibitory effect of FAP positive fibroblasts in patients with high tumour infiltration of CD8 positive T cells on response to platinum-based treatment in a population of HGSOC patients. On the same population, we studied the macrophage profile and discovered a correlation of two distinct subtypes of macrophages (CD11c and CD80 positive) in specific tumour localizations, with overall and progression-free survival respectively, indicating independent effects of these subsets on natural course of the disease and response to treatment.

In summary, our research identified a number of tumour stroma-related measurable features associated with survival and response to treatment. Our findings support continued analyses of ovarian cancer tumour microenvironment in order to discover and develop new prognostic and predictive tools, to improve the clinical outcome in ovarian cancer.

LIST OF SCIENTIFIC PAPERS

I. **Corvigno S.,** Wisman G.B.A., Mezheyeuski A., Van der Zee A.G.J., Nijman H.W., Åvall-Lundqvist E., Östman A., Dahlstrand H. (2016). **Markers of fibroblasts-rich tumor stroma and perivascular cells in serous ovarian cancer: inter- and Intra-patient heterogeneity and impact on survival.** *Oncotarget 7(14),* 18573-18584.

II. Frodin M., Mezheyeuski A., **Corvigno S**., Harmenberg U., Sandström P., Egevad L., Johansson M., Östman A. (2017**). Perivascular PDGFR-β is an independent marker for prognosis in renal cell carcinoma**.
British Journal of Cancer 116, 195-201.

III. **Corvigno S.,** Frodin M., Wisman G.B.A., Nijman H.W., Van der Zee A.G.J., Jirström K., Nodin B., Johansson M., Dahlstrand H., Mezheyeuski A.*, Östman A.* (2017).
Multi-parametric profiling of renal cell, colorectal and ovarian cancer identifies tumor-type-specific stroma phenotypes and a novel vascular biomarker.
Submitted

IV. **Corvigno S**., Mezheyeuski A., Carlson J.W., Fernebro J., Klein C., Åvall-Lundqvist E., Östman A., Dahlstrand H. (2017)
FAP+ cancer-associated fibroblasts affect platinum-response in a subset of high-grade serous ovarian cancer with high T-cell infiltration.
Manuscript

V. Corvigno S., Mezheyeuski A., Carlson J.W., Fernebro J., Åvall-Lundqvist E., Rolny C., Östman A*., Dahlstrand H*. (2017)
Marker- and localization-defined subsets of CD68-positive cells show specific associations with prognosis and response to treatment in high-grade serous ovarian cancer.
Manuscript

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LIST OF ABBREVIATIONS

α-SMA	α-Smooth muscle actin
ADP	Adenosine diphosphate
Akt	Serine/threonine specific protein kinase
BMP	Bone morphogenetic protein
BRAF	Murine sarcoma viral oncogene homolog B
BRCA	Breast related cancer antigens
CA125	Cancer antigen 125
CAF	Cancer-associated fibroblast
CCL	CCR-like protein
CCNE1	Cyclin E1 coding gene
CCR	Chemokine receptor
CD	Cluster differentiation
CI	Confidence interval
CLIC	Chloride intracellular channel
CR	Complete response
CXCL	Chemokine (C-X-C motif) ligand
CyTOF	Cytometry by Time-of-Flight
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethilenediaminetetraacetic acid
EGF	Epithelial growth factor
EGFR	Epithelial growth factor receptor
EMT	Epithelial-mesenchymal transformation

EOT	End of treatment
FAK	Focal adhesion kinase
FAP	Fibroblasts activating protein
FFPE	Formalin-fixed paraffin-embedded
FGFs	Fibroblasts-growth-factors
FIGO	International Federation of Gynecology and Obstetrics
GDF15	Growth/differentiation factor 15
GOG	Gynecologic Oncology Group
GCIG	Gynecological Cancer Intergroup
H&E	Hematoxylin and eosin
HGF	Hepatocyte growth factor
HIF	Hypoxia-induced factor
HPNCC	Hereditary non polyposis colorectal cancer
HR	Hazard Ratio
IFN	Interferon
IGF	Insulin growth factor
IL	Interleukin
INOS	Inducible nitric oxide synthase
IQR	Interquartile range
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGSOC	Low-grade serous ovarian cancer
MAb	Monoclonal antibody
MDSC	Myeloid-derived suppressor cells
MEK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase

MVD	Micro vessel density
Nf-kb	Nuclear factor kb
NG2	Neural/glial antigen 2
NK	Natural killer
OD	Optical density
ORR	Objective response rate
OS	Overall survival
PARP	Poly-ADP ribose polymerase
PD	Programmed cell death protein
PD	Progression of disease
PD-L	Programed cell death protein ligand
PDGF	Platelets-derived growth factor
PDGFRβ	Platelet-derived growth factor receptor b
PFS	Progression-free survival
pH	Potential of hydrogen
PI3K	Phosphoinositide 3 kinase
PKC	Protein kinase C
PLGF	Placental growth factor
PR	Partial response
PTEN	Phosphatase and tensin homolog
PVI	Perivascular intensity
RAD51C	DNA repair protein recombinant A homologous C
RECIST	Response evaluation criteria in solid tumours
RGS5	Regulator of G-protein signalling 5
RNA	Ribonucleic acid

SD	Stable disease
SDF1	Stromal cell-derived factor 1
TAM	Tumour-associated macrophage
TGFß	Tumour growth factor ß
Th	T helper
Tie2	Tyrosine kinase with Ig and EGF homology domains
TILs	Tumour-infiltrating lymphocytes
TMA	Tissue microarray
TME	Tumour microenvironment
TNFα	Tumour necrosis factor a
Treg	T-regulators
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor
WNT	Wingless-related integration site

1. OVARIAN CANCER

1.1 EPIDEMIOLOGY

Ovarian cancer is the seventh most common malignancy in women worldwide, with around 250.000 women diagnosed every year and 150.000 deaths [1]. It is the most lethal among the gynaecological malignancies in developed countries. The relative 5-year survival across all stages in Europe is 38% [2] and 43% in Sweden [3]. Northern Europe has the highest incidence with 10 cases per 100.000 women and in Sweden around 700 women are diagnosed with ovarian cancer per year. The incidence is low below 30 years of age and reaches a peak for women between 65 and 74 years old. Ninety percent of all the ovarian tumours are of epithelial origin, epithelial ovarian carcinomas (EOC). Due to the lack of symptoms when the cancer is localized, most women are diagnosed when the tumour has spread outside the pelvis (FIGO stage III-IV).

1.2 HISTOPATHOLOGY

EOC encompasses five distinct subtypes: high-grade serous, low-grade serous, endometrioid, clear cell and mucinous subtype [4]. High-grade serous ovarian carcinomas (HGSOC) account for approximately 70% of ovarian epithelial carcinomas [4]. Until a few years ago, the old three tiers grading system divided serous ovarian cancers into high, moderate and low differentiated tumours, but since 2012 a new two grading system is used, classifying serous ovarian cancer into low- and high- grade subtype [5] [6]. The two low-grade and high-grade types represent distinct different subtypes with specific molecular and clinical characteristics [7] [8]. Furthermore, the origin and the genetic mutation patterns allow EOC to be divided in type I and type II tumours [9]. Type I tumours are represented by low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas, that are thought to develop in a stepwise fashion from precursor lesions. Type II tumours, instead, are represented by HGSOC, high-grade endometrioid carcinoma, malignant mixed mesodermal tumours (carcinosarcomas) and undifferentiated carcinomas, which grow rapidly and are highly aggressive [10].

1.3 HIGH-GRADE SEROUS OVARIAN CANCER

HGSOC are the prototype of type II lesions and are thought to originate from ovarian epithelium, distal fallopian tube or peritoneum [10]. P53 dysfunctions caused by mutations or post-translational modifications are almost ubiquitous in this type of tumours. Mutations in the TP53 gene have been shown in 95% of these tumours and in the remaining cases p53 dysfunction is caused by posttranslational mechanisms [10] . The existence of a pre-tumoral lesion found in the tuba, called serous tubal intraepithelial carcinoma (STIC), characterized by the so called ”p53 signature”, is the reason for considering distal tuba as the site of origin of most of the HGSOC.

A second genetic marker for HGSOC is BRCA pathway dysfunction which affects half of the cases [11] and can be represented by germline mutations, somatic mutations, or epigenetic silencing. Germline mutation of BRCA, according to the Cancer Genome Atlas Research Network study, were seen in 17% of the HGSOC, while somatic mutations of BRCA in the 3%; in other 11% BRCA1 was epigenetically silenced [11] [10]. In general, defective homologous recombination DNA repair systems can account for different alterations that go under the name of ”BRCAness” . Other altered pathways involve RAD51C, EMSY, PTEN Retinoblastoma [12], PI3K/Akt and Notch signalling cascades can be often altered as amplifications of the cell cycle regulator cyclin E1 gene (CCNE1) [13].

1.4 RISK FACTORS

The most important known risk factors for EOC are age, hereditary mutations, null-parity, certain gynaecological conditions like endometriosis, and hormonal replacement treatment.

A family history of ovarian cancer of having one affected first-degree relative diagnosed with ovarian cancer is associated with a 2 to 3 fold increased risk of being diagnosed with ovarian cancer [14]. At certain high risk of developing EOC are women with breast cancer susceptibility protein (BRCA) 1 and 2 germline mutations. For carriers of BRCA1 mutation the risk of developing EOC before the age of 70 is around 30-40% [15] [16]. The corresponding risk for BRCA 2 mutation is 10-20%. Women with BRCA 1 and 2 mutations [17] are recommended to consider prophylactic surgical removal of ovaries and tubes after childbearing age. Although the inherited syndromes that involve mutations in mismatch repair genes, like hereditary non-polyposis colorectal cancer (HPNCC) (also named the Lynch syndrome), [18] mostly affect the risk of colon cancer and endometrial cancer, women with Lynch syndrome also have a 12% risk of developing EOC [19] [20].

Women with endometriosis have an increased risk of EOC, especially of the clear cell and endometrioid sub type of EOC [18] [21].

Hormone replacement therapy based on oestrogen with or without progestin, slightly increases the risk of ovarian cancer. A Danish prospective cohort study of hormone replacement therapy users reported an EOC incidence rate ratio (RR) of 1.44 (95% CI 1.30-1.58) compared to never users [22]. The protective effect of oral contraception increases with duration of use (6% reduction risk per year) [23] and the risk reduction seems to persist for more than 30 years after ceased use [24]. Oral contraception may in fact be used as chemoprevention for BRCA1/2 mutation carriers [25].

In a meta-analysis conducted on 47 studies, it emerged that the relative risk of ovarian cancer was increased with the increase of both height and body mass index [26]. Parity [20] and breastfeeding [20], [27] can be considered as protective factors. A full-term pregnancy can reduce the risk of developing ovarian cancer up to one third, and additional pregnancies reduce the risk further [20] .

1.5 STANDARD PRIMARY TREATMENT

Primary surgery is the cornerstone of treatment for EOC. Surgery includes removal of uterus, tubes, ovaries, omentum, biopsies of peritoneum and all visible tumour lesions. Pelvic and para-aortal lymph node removal is performed when pre-operative suspected stage I (except for mucinous subtype). Advanced stage disease often requires a more extensive surgery with multiple bowel incisions, splenectomy and stripping of the peritoneal surface in order to obtain macroscopic radical surgery. If the extents of the disease don´t allow a proper debulking, the surgery can be preceded by two to four cycles of neoadjuvant chemotherapy (the surgery is then named delayed primary or interval surgery) [28].

Only patients with confined disease stage IA-B and certain histology subtypes, are subjected to surgery without any adjuvant chemotherapy. The great majority of patients receive chemotherapy after surgery [29]. The standard treatment is a combination of platinum and taxanes [30]. Current standard post-operative chemotherapy consists of six cycles of carboplatin-paclitaxel [31] [32]) administered intravenously every three weeks. According to recent evidences [33], [34] bevacizumab, a monoclonal antibody against VEGF, is approved in combination with paclitaxel and carboplatin, followed by mono-treatment in maintenance regime for 12-15 months, in primary treatment of epithelial ovarian cancer stage IIIC with presence of residual tumour after debulking surgery, or in stage IV.

Resistance to therapy is a major problem, since approximately 75% of advanced epithelial ovarian cancers will relapse within three years [35]. According to treatment sensitivity, tumours can be classified in refractory, resistant and sensitive to platinum, on the basis of the time interval between the last course of platinum and relapse or progression, called the platinum-free Interval (PFI). Relapse/progression after 6 months of PFI categorizes a patient as platinum sensitive, and these patients will likely respond to a second line of platinum treatment. A tumour progressing on platinum based therapy in less than 6 months from the last course is classified as resistant, associated with worse prognosis. A tumour not even initially responding but relapsing or progressing during the platinum treatment is considered refractory.

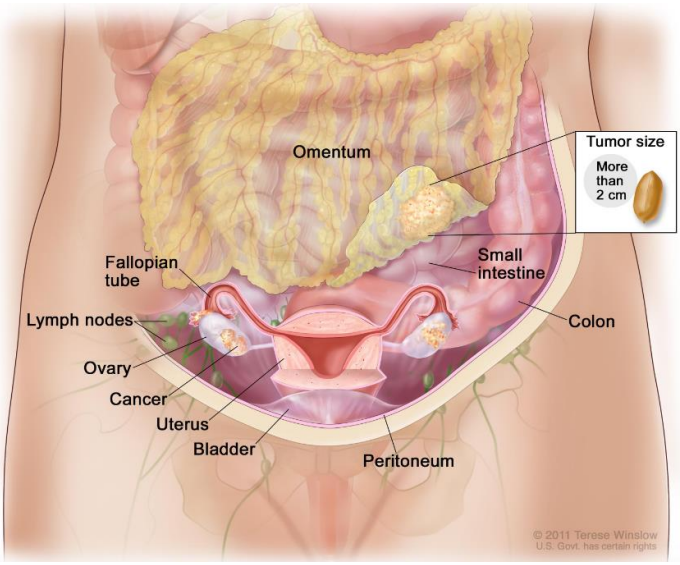

Figure 1: Ovarian cancer FIGO stage IIIC. The cancer is found in one or both ovaries and has in this case spread to the omentum (> 2 centimeters) and the peritoneum. The cancer may have spread to lymph nodes behind the peritoneum, or to the surface of liver or spleen. *Figure adapted from National Cancer Institute (NCI) website https://goo.gl/images/SQBJNi*

1.6 PROGNOSTIC FACTORS AND MARKERS

Few prognostic biomarkers, and clinical markers, have been established for EOC mostly due to the heterogeneity of the disease. The most important predictor of survival is stage at diagnosis. Overall survival at five years for EOC range from 90% in stage IA to 20% in stage IV [36]. Age, histology, grade and residual disease after surgery are all established significant prognostic clinical factors for ovarian cancer [37]. Regarding prognostic biomarkers, a few of

them have so far been established. Currently there is no biomarker fully accepted and recognized to guide the choice of therapy in ovarian cancer. Cancer Antigen 125 (CA 125) is a valuable serum biomarker for tumour detection and monitoring [38]. Positive oestrogen receptor or progesterone receptor expressions have been associated with improved survival in endometrioid carcinoma [39]. Serum VEGF and PDGF have been recently studied as prognostic biomarkers, with some encouraging results. Elevated serum VEGF levels have been associated with poor progression-free survival (PFS) (HR 2.46, Cl 95% 1.84-3.29), showing a higher impact in the early stages [40, 41]. The different prognosis shown by HGSOC has aroused questions about the existence of different molecular subtypes in this class of ovarian cancers. Verhaak and colleagues [42] used a dataset from 489 HGSOC included in the Cancer Genome Atlas Research Network study [11] to develop gene signatures related to different subtypes with different prognosis. The gene signatures were divided into subtypes called: differentiated, immunoreactive, mesenchymal and proliferative, with the immunoreactive group having the best prognosis and the mesenchymal one having the worse prognosis.

1.7 PREDICTIVE MARKERS

HGSOC patients with BRCA mutations, especially BRCA 2, have been shown to have a higher response rate to chemotherapy and a better survival in general [43]. Furthermore, BRCA mutations are the main predictive marker for PARP inhibitors therapy, due to the ”synthetic lethality”.

In conclusion, there is an impelling need for the search of new prognostic and predictive tools to be included in the management of ovarian cancer. The supportive stromal network, underling and surrounding tumour cancer cells, has been speculated to be a source of available biomarkers both for survival and response to therapy prediction.

2. TUMOUR MICROENVIRONMENT

2.1 INTRODUCTION

The malignant cancer cell has been the principal topic over the last forty years of cancer research, and researcher used to believe in its total centrality in the biology of cancer. In the last few years a new vision of the process of tumour initiation and progression has taken place, involving the role of what is now called the “tumour microenvironment” or the “tumour stroma”. Composed of different kinds of cells, the tumour stroma creates a

permissive environment for cancer, vital for its growth and spread and helps in several established cancer hallmarks [44]. Currently the tumour microenvironment represents a broad and fast moving field, with influences on prognosis and sensitivity to therapy [45, 46]. The most important components of the tumour stroma are cancer-associated fibroblasts, endothelial cells, perivascular cells, referred to as pericytes, and immune cells. The next paragraphs we will focus mostly on these four cell types.

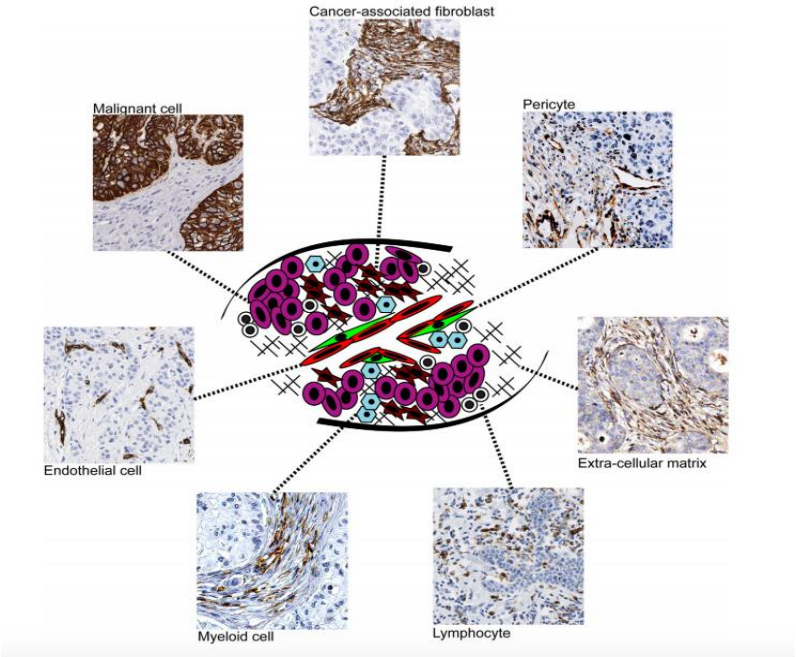

Figure 2: A schematic cartoon portraying the various constituent cell types within tumor microenvironment. *Figure adapted from review of Pietras and Östman [44].*

2.2 CANCER-ASSOCIATED FIBROBLASTS

2.2.1 GENERAL BIOLOGY

Cancer-associated fibroblasts (CAFs) are large spindle-shaped cells, considered, broadly as activated fibroblasts within the tumour stroma expressing different myofibroblastic markers [47]. The origin of CAFs is under debate, but a few alternative sources seem to be the most validated ones: resident fibroblast; epithelial or endothelial cells in trans-differentiation; mesenchymal cells such as vascular smooth-muscle cells, pericytes or adipocytes; and finally bone marrow derived precursors [47].Their different origins can be translated into different subtypes possibly contributing in different ways to the promotion of tumour growth and

invasiveness.

2.2.2 TUMOUR INITIATION

CAFs can express and secrete signalling molecules, for example mithogenic epithelial growth factors as hepatocyte growth factor (HGF), epidermal growth factor (EGF), insulin like growth factor 1 (IGF-1), stromal cell derived factor 1 (SDF-1/CXCL12), fibroblast growth factors (FGFs) that stimulate cancer cells proliferation [48] [49], contributing to the process of cancer initiation. They can also secrete proinflammatory mediators that recruit immunitary cells, which in turn provide mitogenic signals to cancer cells [50] [51]. Erez et al. described in 2010 a proinflammatory gene signature in CAFs isolated from dysplastic skin. The signature was seen also in breast and pancreatic cancers and marked a pathway that promoted macrophage recruitment and tumor growth, dependent mostly on the Nf-kb signalling. These proinflammatory genes could be expressed by dermal fibroblasts under stimulation by carcinoma cells [52].

2.2.3 STEM CELLS SUPPORT AND EPITHELIAL TO MESENCHYMAL TRANSITION (EMT)

Vermeulen et al. observed in colon cancer that the Wingless/Integrated (WNT) pathway in cancer cells, needed to maintain cell stemness, was particularly active in tumour cells located close to stroma fibroblasts [53]. They demonstrated that HGF secreted by tumour stroma myofibroblasts activates beta-catenin dependent transcription and cancer stem cells clonogenicity. The stem cell phenotype could, in this way, also be rescued in more differentiated tumour cells [53]. CAFs are also involved in the process of epithelial to mesencymal transition (EMT) via secretion of TGFβ [54]; they are able to limit cancer cell apoptosis [49] [44], in one way secreting IGF-1 and 2 as survival factors, and in a second way secreting extracellular matrix (ECM) remodelling proteases that provides non diffusible survival signals.

2.2.4 IMMUNE MODULATION

The evasion of immune control is another important hallmark capability of tumor cells. Preclinical studies suggest that fibroblast activating protein (FAP) positive stromal cells are able to induce escape from immunological control by CD8+ T cells. In a model of Lewis lung carcinoma it was demonstrated that ablation of FAP positive fibroblasts was able to induce hypoxic death mediated by TNFα and IFNγ (normally involved in CD8 positive T cell-dependent killing) [55]. Another mechanism of escaping immunological control involves the

inhibition of cytotoxic T cells and NK/T cells, through TGF β production, as shown by Stover et al. in 2007 [56]. Zahng et al in 2016 demonstrated that depletion of FAP+ fibroblasts could reduce the metabolic stress of tumour infiltrating lymphocytes (TILs) [57].

2.2.5 METABOLIC INTERACTION WITH CANCER CELLS

CAFs can modulate the energetic metabolism of cancer cells. After stimulation by reactive oxygen species secreted by cancer cells, CAFs can switch on aerobic glycolysis and produce lactate and pyruvate which in turn can be used as fuel by tumour cells [58]. A model proposed by Martinez-Outschoorn et al. considers the existence of a "two compartments tumour metabolism" where tumour stroma cells are catabolic and tumour cells are anabolic, and the energy is transferred from the catabolic compartment to the anabolic one through sharing of nutrients [59].

2.2.6 METASTASIS

Activated fibroblasts can control invasiveness and metastasis through different mechanisms. First of all, they can trigger angiogenesis promoting tumour vasculature formation resulting in improved tumor growth and spreading. CAFs can produce proangiogenic factors like VEGF, FGFs, IL-8/CXCL8 and PDGF-C. For the same purpose they produce ECM degrading molecules that release angiogenic factors [60], or chemo attractants for other proangiogenic myeloid cells [61]. Fibroblasts have moreover been identified as providers of pro-metastatic signalling in a paracrine and in a systemic fashion. Karnoub has showed in 2007 that secretion of CXCL5 from mesenchymal stem cells previously stimulated by breast cancer cells, can enhance in a paracrine way their invasiveness and metastatic potential [62]. The metastatic colonization in distant sites is another capability that cancer cells should acquire in order to be able to form metastasis. The process of spreading and colonize distant organs is not always successful since only a minority of tumour cells that reach distant sites can actually settle and form metastatic colonies. Malanchi et al. in 2011 have described the crucial role of stromal niche signals in the secondary target organs in the process of metastasizing [63]. They identified periostin, produced by fibroblasts, as required for cancer stem cell maintenance in distant organs and showed that its blocking could inhibit metastasis formation. Several factors are produced and secreted systemically by activated fibroblasts in order to enhance the metastatic potential of cancer cells; two of these are represented by HGF, the c-Met ligand, that stimulates invasiveness and proliferation and TGF β that enables cancer cells to invade [54]. Another example of systemic effects promoted by CAFs in the primary tumour site, is the expression of GDF15, a TGFβ/BMP family member, by prostate

cancer-associated fibroblast, which not only exerts paracrine stimulation of tumour cells growth and migration, but also instigates their growth in distant sites [64].

2.2.7 PROGNOSTIC IMPACT

Several studies have shown a connection between markers expression of stroma fibroblasts and prognosis in different cancer types, like prostate and breast tumours [65].

Prognostic studies used different techniques to detect biomarkers, focusing either on protein expression or on mRNA expression.

Two studies recognized tumour growth factor β (TGFβ), detected at a protein level, to be related to tumour progression in non-small cell lung cancer [66] [67]. Again in lung cancer, platelet derived growth factor A (PDGFA) expression, detected with immunohistochemistry on tumour samples, correlated with lymph node metastasis, while co expression of VEGFR-3 and PDGFB was an independent indicator of poor prognosis [68] [69].

One of the first studies to use gene expression analysis in order to explore potential stroma related signatures was performed by Chang et al in 2004. They detected a gene expression profile of fibroblasts from several anatomic sites, which was stereotyped and occurred after serum exposure [70]. A microarray analysis performed on normal fibroblasts and cancer associated fibroblasts derived from non-small cell lung cancer, revealed around 11 differentially expressed genes that formed a prognostic gene expression signature, validated then in clinical datasets [71] Frings et al. described a gene expression signature expressed by PDGF activated fibroblasts able to identify a breast cancer subset with a specific prognosis pattern [72]. The PDGF signature score proved to be associated with clinical characteristics such as human epidermal growth factor 2 positivity, oestrogen receptor negativity, high tumour grade and large tumour size. The signature proved to be related with shorter survival [72].

Recently a growing body of evidence leans in favour of the existence of multiple cancer-associated fibroblasts subtypes behaving in different way sin relation to cancer cells. Ozdemir et al in 2014 showed in a mouse model of pancreas cancer that targeting α-SMA positive myofibroblasts lead to a more invasive tumour phenotype [73]. CAFs depletion, in this study, generated increased hypoxia, enhanced epithelial to mesenchymal transition and determined reduced survival of the animals.

2.2.8 RESPONSE TO THERAPY

CAFs have recently been shown to be involved in modulating sensitivity of cancer cells to therapy. Sun and colleagues have shown that the expression of a component of the Wnt family secreted by fibroblasts in the tumour microenvironment of prostate cancer was responsible for attenuation of chemotherapy response in vivo [74]. More recently it was proven the ability of CAFs to modulate sensitivity of tumour cells to new target therapies. Straussman and colleagues in 2012 characterized a stroma-derived pattern of resistance of BRAF mutated melanoma cells to RAF inhibitors [75]. Subsequent proteomic analyses identified the responsible secreted factor as HGF, acting on MET receptor and activating the AKT pathway. New evidences show influence of CAFs on response to endocrine treatment for breast cancer [76]. A new intriguing theory suggests that stromal cells can influence breast cancer cells sensitivity to treatment through paracrine and juxtacrine signalling upon exosome transfer [77]. Another study found an association between loss of stromal caveolin 1 and poor clinical outcome in breast cancer suggestive of tamoxifen resistance. This feature in the subgroup of lymph node positive patients was related to an 11.5 fold reduction of 5- years progression free survival [78]. A recent work from Paulsson et al showed that high expression of stromal PDGFRß confers reduced sensitivity to adjuvant tamoxifen, in two large cohorts of women affected by breast cancer [79]. These evidences constitute the basis to investigate new therapeutic approaches to target cancer-associated fibroblasts.

2.3 ENDOTHELIAL CELLS

2.3.1 GENERAL BIOLOGY OF ANGIOGENESIS

The so called ”angiogenic switch” is a well-known trigger for tumour growth, because it increases cancer cells proliferation and sustains the real formation of the tumour mass [80]. Also well known is that inhibiting this switch can result in arresting tumour growth possibly because of the reduced flow of mitogenic growth factors usually provided through the blood flow [81] [82]. Another important role of tumour vessels is to provide oxygen, besides survival factors, needed by the tumour cells to escape cell death. In fact, induction of apoptosis and necrosis are inevitably a result of destruction of tumour vasculature. Vascular disrupting agents cause acute hypoxia and massive cell death [83]. Angiogenesis is physiologically activated in the embryonic phase and in the female reproductive cycle, otherwise it is triggered just in some pathological conditions such wound healing in which tissue remodelling is needed [82], and in cancer. During tumour formation, angiogenesis occurs with the same mechanisms used in physiological conditions, but it is constitutively

activated and lacks of the “normal” regulation, resulting in the formation of irregular, chaotic and unstable vessels [84] [85]. Stromal cells have an active part in orchestrating of the tumoral angiogenic process. Immune cells are recruited by endothelial cells and interact with them producing several soluble factors implicated in regulation of angiogenesis like cytokines (VEGF, bFGF, TNF alpha, PDGF and PIGF), chemokines (CXCL 12, IL 8/CXCL8), matrix metalloproteinases (MMP 2-7-9-12-14), serine proteases, cysteine cathepsin proteases, DNA-damaging molecules (reactive oxygen species ROS), histamine and nitric oxide. These molecules are fundamental in regulating vessel cells proliferation, survival, motility and vessels formation [86]. A class of macrophage renamed as ”tumor associated macrophages (TAMs)” is known to regulate angiogenesis through VEGFA production [87]. Stockmann et al in 2008 showed how genetic deletion of VEGFA in macrophages determines attenuation and ”normalization” of tumour angiogenesis [88]. Another factor produced by TAMs that activates and stimulates angiogenesis in some tumours is the VEGF family member PIGF [89].

2.3.2 METASTASIS

Angiogenesis has a prime role in cancer cells dissemination and metastatization. VEGF was first identified as vascular permeability factor that through VEGFR2, loosens tight junctions making vessels permeable to leakage of blood in the interstitial sites and to cancer cells to enter the circulation. Another mechanism that enables endothelial cells to increase vascular permeability is through activation of CCR2+ which responds to CCL2 secreted by tumour cells; CCR2 deficiency, in fact, prevents colon carcinoma cells extravasation and metastatic dissemination [90]. Hypoxia around vessels also contributes to metastatic dissemination through the activation of genes regulated by the hypoxia inducible factor HIF, and the inducible nitric oxide synthase iNOS [91]. About distant metastatization it is now believed that primary tumours with metastatic potential can precondition vasculature in metastatic sites via production of factors such as VEGF, provided systemically. Newer evidences show that stimulation of Notch signalling in endothelial cells by tumour cells, can modify endothelial cells morphology and function in a way that stimulates cancer cell migration, invasion and settling in distant sites [92].

2.3.3 THERAPEUTIC IMPLICATIONS

With these premises the concept of antiangiogenic cancer therapy seems simple and straightforward, but its goal should not only be the one of destroying tumor vasculature depriving tumor of its nourishment. In fact better clinical outcomes were seen when

antiangiogenic drugs have been administered together with chemotherapy or additional drugs [93]. An example of that is VEGF-A/ VEGF 2 blockage that determines transient vessel remodelling and normalization with increased pericyte coverage, resulting in reduced permeability and facilitated drug access [94] [95]. Tavora et al. propose a mechanistic model followed by a clinical translation of the same model in which deletion of focal adhesion kinase (FAK) in endothelial cells is sufficient to induce tumour cell sensitization to DNA damaging therapies in mice. The clinical observation of low blood vessels expression of FAK being associated with complete remission in patients affected by lymphoma supports this hypothesis [96]. A more extended paragraph about endothelial cells as target of new therapeutically approaches will follow in chapter 3.1.4.

2.4 PERICYTES

2.4.1 GENERAL BIOLOGY

Pericytes are defined as the class of cells that surround endothelial cells, constituting part of the mural vessel layers. They are usually covered in the same basement membrane as the endothelial cells [97], and they can derive from mesoderm, neural crest, bone marrow or endothelial cells themselves. They are described as fibroblasts-like with long protrusions through which they take contact with endothelial cells, and can be classified in precapillary pericytes, mid capillary pericytes and post capillary pericytes. These cells have recently shown to express a wide range of markers that might change according to the different times of their development and the different tissues they belong to. Among those, the most known ones include PDGF receptor beta (PDGFRβ), alpha smooth muscle actin (α-SMA), desmin, NG2, and regulator of G protein signalling 5 (RGS 5) [98] [99]. Tumor pericytes are characterized by a more loose attachment to endothelial cells and by a specific pattern of activated signalling pathways [100]; more of them are involved in their recruitment and differentiation like PDGF, transforming growth factor beta (TGF beta), angiopoietin and Notch family members. Recruitment of pericytes into tumours and blood vessels is dependent on PDGF signalling. Indeed pericytes express PDGFRβ, which is activated by PDGF usually produced by endothelial cells [101].

2.4.2 METASTATIC PROCESS

Since one of the most important external regulators of initial phases of tumour growth is neovascularization through tube forming, the supporting pericytes play a fundamental role in tumour initiation [102]. Their role in tumour growth and spreading though is still yet to be

fully elucidated. In B12 melanoma model, the induced up-regulation of PDGF expression in cancer cells resulted in an increased pericytes proliferation with enhanced tumor growth (despite a normal vessel density) [103]. When PDGF BB ligand overexpression was induced in colorectal and pancreatic models, the increase of pericytes coverage was linked to an inhibition of tumour growth [104]. The same contrasting effects have been noticed when studying the role of pericytes in the metastatic process. Some evidences show that genetically pericytes-poor RIP1-Tag2 mice have an increased rate of metastasis from pancreas lesions [105]. In a model of breast cancer, it was shown by Keskin et al that depletion of PDGFRβ positive pericytes in early stages of tumour development resulted in decrease of tumour growth, while depletion of the same cells in advanced setting, significantly increases lung metastasis [106]. Contrasting evidences are offered by Yang and colleagues, when showing that a paracrine interaction between perivascular cells and TAMs promotes tumour metastasis through the IL-33-ST2-dependent pathway, in tumour xenografts [107]

2.4.3 PROGNOSTIC IMPACT

In a broader perspective, pericytes have a role in increasing tumour aggressiveness allowing an immune-permissive tumour environment. Hong J et al proved in 2015 that a paracrine communication between pericytes and inflammatory cells promotes recruitment of immune suppressor MDSC cells in tumour site leading to increase in tumour growth and circulation of malignant cells [108].

Sinha and colleagues in 2016 provided in vitro and in vivo evidences, supported by large human database analysis, that pericytes were able to accelerate tumour growth in xenografts models. Moreover, a pericytes mRNA signature was able to predict poor prognosis in two serous ovarian cancer patient datasets [109].

In two cohorts of patients with clear cell renal cancer, high α-SMA positive pericytes coverage, was found to be associated with a poor outcome by Cao et al [110].

Lately our group has produced some evidences in favour of the prognostic role of perivascular cells in survival, in colorectal, renal and ovarian cancer [111] [112, 113].

2.4.4 RESPONSE TO THERAPY

Pericytes have the ability to modulate the efficacy of tumour treatments. It has been shown that they can induce survival signals in endothelial cells providing the rational for dual targeting of both kinds of cells [114, 115]. Moreover, pericytes can impair immune-

modulating therapies through regulation of immune cell trafficking [116]. Finally, pericytes have been shown by Armulik et al. to regulate some important functions of the blood brain barrier; pericytes deficiency in this study caused increased permeability of the barrier to low and high molecular mass tracers [117].

A higher coverage of α-SMA positive pericytes was also related to a better response to neoadjuvant bevacizumab in a cohort of breast cancer patients undergoing a phase II trial with preoperative anti-angiogenic therapy [118].

2.5 IMMUNE CELLS

2.5.1 GENERAL BIOLOGY IN TUMOUR MICROENVIRONMENT

Cancer immune surveillance has been a debated argument in tumour biology since the beginning of the century. The difficulty in finding proper animal models in order to test the hypothesis that the immune system is involved in cancer development has represented an obstacle just recently removed. The use of transgenic mouse technology allowed to define a dual role of the immune system in tumour development: protection from cancer development and promotion of it [119] [120].

The continuous in vivo investigation culminated in the evidence of the impact of immune cells on prognosis of human tumours [121]. A fundamental step in the investigation of the immune microenvironment was the analyses of the in situ immune components that constitute the tumour immune infiltrate.

During early tumour development, immune cells start to invade the area; all immune cell types participate to this activity: macrophages, dendritic cells, mast cells, natural killers (NK) cells, B lymphocytes and effector T cells, including regulatory T cells (T reg) and cytotoxic T cells [122]. Different types of immune cells can be found in different areas of the tumour and in different stages of tumour development [123]; this suggests the existence of distinct roles for immune cells in tumour control, and clinical outcome.

CD8 positive cytotoxic T lymphocytes have been generally associated with a good outcome in several tumour types, as in ovarian [124] [125], colon [126] [127], and breast cancer [128] [129].

T reg cells, in general detected by the expression of CD4 and characterized by an immune suppressor phenotype, have mostly been related with poor prognosis, in ovarian cancer

[130] and breast cancer [131]. The difficulties in detecting this subtype of T cells has brought to discrepancies in results of studies on their prognostic significance [132].

NK cells have generally a good impact on prognosis, as shown for colorectal, gastric, lung and renal cancer [133] [134, 135], when detected by CD57.

Infiltrating B cells can have an antitumor effect in breast and ovarian cancers [136] [137], but their effect has not yet been determined for most of the other cancer types.

Tumour microenvironment interacts with T lymphocytes in different ways but this communication mostly brings to immune-suppression. This has been clear after the discovery that systemic immune response can fail to produce a valuable suppressive response in human cancers [138]. In this study, Schreiber et al show that the presence of specific CD8+ T lymphocytes recognizing the antigen expressed by melanoma cells, does not prevent progression of melanoma itself. Different events have been suggested to generate this immune suppressive effect, one of this being physical exclusion of T CD8+ cells from vicinity of cancer cells as suggested in colon [121], ovarian [125] and pancreas cancer [139]. The latter study showed that the major players of this interaction are cancer-associated fibroblasts, although tumour vessels and endothelial cells can contribute to regulate extravasation, and accumulation of T effector cells in the tumoral sites [140]. The study also revealed that FASL (FAS ligand) is induced by VEGF in tumour vasculature, causing an apoptosis-mediated cell death of T cytotoxic cells (T reg might be protected by their high expression of apoptosis inhibiting proteins).

In ovarian cancer, high expression of VEGF and CD267 (an immunoregulatory molecule) from the tumour vasculature is related to low infiltration of T cells and worse clinical outcome [124] [141].

Another mechanism through which tumour microenvironment cells suppress T cell immunity is via the PD-1- PD-L1 axis. PD-1 antigen is expressed by T lymphocytes and limits their activity in peripheral tissues [142] [143]. When PD-1, which is expressed by T cells due to their activation, is recognized by some of his ligands, the kinases related to T cell activation are inhibited. The two most known ligands for PD are PD-L1 and L2 which have been found expressed mostly on cancer cells and myeloid cells [144] [145]. Therapies targeting this pathway have been positively associated with good results in several tumour types [146].

In such a heterogeneous environment, the balance between immune promoting and immune suppressing factors can influence immune cells phenotype and determine the outcome of the immune response. A valid example of that is the plasticity of macrophages. Macrophages are supposed to originate from circulating precursors [147], myeloid cells and myeloid derived-suppressor cells [148]. Macrophages have different functions in the immune infiltrate that go from increasing the antigen availability to its clearance, and are modulated by a complex system of cytokines [149]. . They can polarize into different subsets expressing different patterns of cytokines, enzymes and markers. Many refer to polarized macrophages as ”M1” and ”M2” following the general distinction of T cells in Th1 and Th2. According to this very general distinction, the M1 phenotype is driven by Th1 cytokines like interferon-γ and produces pro-inflammatory factors like IL-6, IL-12, IL-23, and TNF-α; moreover, they express high levels of histocompatibility molecules class II and I. M2 macrophages are instead supposed to be anti-inflammatory and pro-tumorigenic. IL-4, IL-13, IL-10 guide this phenotype usually during late stage tumour progression [150].

Subtypes of macrophages defined as Tie2 (angiopoietin) - expressing monocytes are supposed to have specific angiopoietic functions [151].

2.5.2 PROGNOSTIC AND PREDICTIVE IMPACT

The definition of the role of tumour-associated macrophages in human cancer prognosis has been complicated by the detection system used. As T cells, macrophages share several membrane markers, expressed at different intensities during different stages of maturation [152]. CD68 has broadly been used as a pan- macrophage marker. In more recent studies different markers have emerged as macrophage-associated, or as polarized macrophage-markers. Two examples are CD163 and CD204, related to the M2-subtype, have been associated with bad prognosis in pancreatic cancer [153]. Another molecule expressed by polarized M2 macrophages is stabilin1, correlated with short survival in colorectal cancer [154].

Association of macrophages with bad prognosis has been widely proven in different tumour types [155] [156], nonetheless in some tumour types like ovarian and gastric, it has been shown the opposite [157, 158]. Similar findings have been published for colorectal cancer [159]. This difference in prognostic significance might mirror a different contribution from different macrophage subpopulations, and suggests the necessity of defining more stringent markers to capture the heterogeneity of macrophage subsets.

Some evidences of interaction between macrophages and chemotherapy treatment derive from lymphoma, and pancreatic cancer. In follicular lymphoma patients with high CD68 positive cells infiltrate in the tumour site benefit of a better survival if treated with doxorubicin-based regimens [160] compared to regimens without doxorubicin [161]. In pancreatic cancer, TAM infiltrate determines higher responsiveness to adjuvant gemcitabine [162]. However, macrophages do not seem to support in general a better response to chemotherapy. Evidences show that M2 macrophages interact with cytotoxic agents increasing tumour-promoting mechanisms [163] [164]. In some cases, M2 macrophages can promote re-growth of tumours after chemotherapy treatment, by triggering re-vascularization [165].

A specific role of subsets of macrophages in response to anti VEGF treatments needs to be mentioned. Macrophages are indeed major producers of VEGF [166]. Hypoxia resulting from vessels destruction can trigger recruitment of macrophages, that implement angiogenesis through alternative mechanisms [167].

2.6 HETEROGENEITY OF TUMOUR STROMA-DERIVED CELLS

Based on the multitude of activities performed by tumour stroma cells, it is easy to deduce that what was some years ago referred to as a homogeneous group of cells, is nowadays considered a collection of distinct subtypes of cells, characterized by a set of distinct markers.

Concerning fibroblasts, candidate markers for these subtypes can be PDGF receptors and FAP, but many more are likely to be soon identified. An useful approach to clarify this issue is represented by new technologies such as CyTOF, in situ gene expression profiling and single cell RNA sequencing [168] [169]. Different subsets of cells can have different origins, and recent studies have addressed this question using the "lineage tracing" technique [170] [171]. The idea that tumor stroma is a heterogeneous and continuously changing pool of cells and matrix, is lately being supported by several publications suggesting the possibility that some kinds of stroma cells could work "against" cancer progression. This is true in particular for CAFs, pericytes and macrophages. In the first case, emerging evidences in pancreas tumour biology, view CAFs as protective against the tumoral development [172] [173], opposite to the old-fashioned view of them as tumour promoting.

About pericytes we have seen how different subtypes, in different tumours, can promote [103] or restrain tumour growth [104]. A similar process can happen within the same tumour at different developmental stages, for what concerns their activity in facilitating or inhibiting metastasis formation [106]. Recent evidences reveal that differential patterns of markers-

expression can characterize, in breast cancer, different subtypes of pericytes potentially connected with different prognosis and sensitivity to therapy [174].

The diversity of tumour-associated macrophages has been widely explored in the previous chapter. Their polarization into different subsets exemplifies the plasticity of the immune microenvironment in relation to tumour development.

These new findings show that tumour microenvironment might be composed of cells undergoing different functional switches during different steps of tumorigenesis. Our new challenge will be to characterize these components and turn our knowledge about their heterogeneity into a new therapeutic weapon.

3. TUMOUR MICROENVIRONMENT IN OVARIAN CANCER

3.1 INTRODUCTION

The progression of tumour cells from transformation to invasion and metastasis, relies on the communication with the surrounding microenvironment.

Ovarian stroma is a functional part of the organ and its role in the normal ovary is well established. The interstitial stroma supports and actively helps and triggers some of the stages of folliculogenesis, up to the ovulation stage [175]. For instance, molecules like BMP 4 and BMP 7 (members of TGF beta family factors), expressed by ovarian stromal cells have been implicated as positive regulators of the primordial to primary follicule transition [176] [177].

As in the normal organ, during tumorigenesis ovarian stroma deeply interacts with ovarian cancer cells through a synergistic paracrine communication involving several cell types and molecules. Although multiple cells are present in the ovarian tumour microenvironment, one of the principal actors dominating the intricate net of signals is represented by CAFs. Some studies have shown how the interaction between ovarian cancer cells and normal fibroblasts can activate the latter to CAFs. Yao et al in 2009 demonstrated that conditioned medium from SKOV3, an ovarian cancer cell line, is able to activate normal fibroblasts into α-SMA expressing cancer associated ones, probably through secretion of a factor called chloride intracellular channel- like 4 (CLIC 4) [178]. Moreover, CAFs from ovarian cancer tissues have been proven to induce migration and invasion of ovarian cancer cells [179].

Fibroblasts are not the only relevant components of ovarian tumour stroma, vessels,

inflammatory cells and ECM components interact to help tumour development and have been described as prognostic indicators [180] [181] [182]. The particular pattern of ovarian cancer metastatization mostly involves spreading in the peritoneal cavity. When ovarian cancer cells migrate around the peritoneal cavity, and it is still unclear if this route is chosen for a simple anatomical advantage or if factors secreted by peritoneal cells and cells of the omental tissues guide ovarian cancer cells through the metastatic process.

There is a growing body of evidence that shows how cell-cell interaction via adhesion molecules and secreted factors are responsible for the homing of ovarian cancer cells through the peritoneal lining [183]. Mesothelial cells can help ovarian cancer cells and sustain them with growth factors such as VEGF and FGF2 [184]. In turn, epithelial ovarian cancer cells can condition mesothelial cells to promote adhesion and invasion through secretion of TGF beta and plasminogen activator inhibitor type I [185, 186].

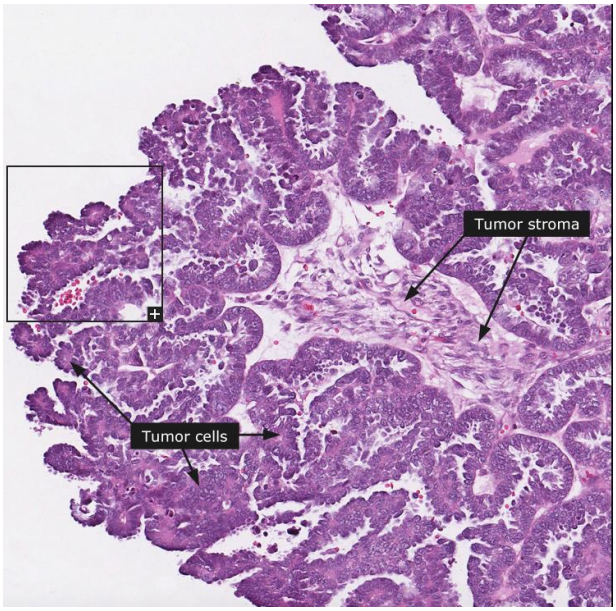

Figure 3: Histology of serous ovarian cancer**.** *Figure adapted from The Human Protein Atlas.*

3.2 STROMA-RELATED PROGNOSTIC FACTORS

Lately, several studies addressed the issues of ovarian stroma compartment being related to prognosis in ovarian cancer patients. Labiche and colleagues, measuring the stroma compartment according to the collagen content, found a negative correlation between survival and amount of stroma in a subset of patients with epithelial ovarian cancer stage III and IV [187]. Moreover a recent study reported that Fibroblasts Activation Protein (FAP) expression

in stroma fibroblasts to be associated with worse overall survival and worse platinum response in a cohort of patients affected by epithelial ovarian cancer [188]. Following this path of investigation, Wimberger and colleagues found a positive association between expression of VEGFR 1 in primary ovarian cancer tissues, found mostly expressed in the vascular wall and across the stroma and decreased progression free survival, again confirming the common opinion that describes stroma and stroma related factors in ovarian cancer to be associated with a poor prognosis [189].

An interesting recent study by the Australian Ovarian Cancer Study Group investigates the association of a pericytes score determined through gene expression data, with prognosis of ovarian cancer and found it related to poor survival in two independent databases. They confirmed their results in in vitro co-cultures, mouse xenografts and patients tissue microarrays expression of α-SMA protein [109].

Recently a study from Horikawa and colleagues, analysed gene expression and protein expression of HGSOC and found that an up regulation of myeloid cells chemo attractants was present in cases where VEGF expression was high, and was associated to shorter survival A mouse model with MDSCs expressing VEGFR1 and 2 helped to understand that migration and differentiation of these cells were stimulated by VEGF, thus confirming the negative role of VEGF on prognosis of ovarian cancer [190]

Microvessel density (MVD) was also studied in relation to tumor prognosis ovarian cancer. Nadkarni et al revealed the existence of a relationship between null mutation rate of p53 and high MVD, detected with CD31 staining, in patients affected by stage III and IV ovarian cancer. The association of high MVD and mutation of p53 was related to increased recurrence risk and worse overall survival [191]. Another study evaluated MVD using fully automatic image analysis, and found it to be an independent prognostic factor in advanced ovarian cancer patients [192].

One of the most important studies to show a contribution of the immune microenvironment in ovarian cancer was published in the New England Journal of Medicine and described a good prognostic effect of intra-tumoral T lymphocytes [124]. Immediately following was the discovery that PD-1/PD-L pathway, involved in the immunosuppressive response, is a prognostic factor for ovarian cancer [193]. Tumour- associated macrophages have not yet been deeply studied in ovarian cancer. A few findings associated them with good prognosis as described in paragraph 2.6.

3.3 STROMA-TARGETED THERAPIES

Many of the new therapeutic approaches in ovarian cancer are addressed to tumour microenvironment and, involve the use of vascular endothelial growth factor (VEGF) pathway inhibitors (antibodies and small tyrosine kinase inhibitors), angiopoietin inhibitors, immune checkpoint inhibitors.

Bevacizumab is an anti-VEGF antibody with antiangiogenic activity that has shown a gain in PFS when added to chemotherapy followed by maintenance in first line, but no overall survival (OS) gain [33]. However, in a subgroup analysis of the ICON 7 trial, the benefit of bevacizumab in terms of overall survival seems to be higher in patients with advanced disease and residual tumour after primary surgery (36.6 versus 28.8 months, HR 0.64, 95% CI 0.48-0.85) [34]. In both platinum sensitive relapse (OCEAN trial) and in platinum resistant relapse (AURELIA trial), adding bevacizumab to chemotherapy yielded an improved PFS [194] [195]. Bevacizumab is nowadays approved for the use in first line as well as for relapse of ovarian cancer.

Other angiogenesis inhibitors are tested in clinical trials. One is the angiopoietin inhibitor is trebananib (AMG 386). In a phase III trial trebananib (TRINOVA-1 trial) plus paclitaxel treated patients experienced prolonged PFS [196].

Pazopanib is a tyrosine kinase inhibitor that targets VEGFR-1, 2, 3, PDGFR α and β, and c-kit. In a phase III study, Pazopanib administered as maintenance after first line standard treatment and showed a gain in PFS of 4.6 months [197].

Cediranib is a molecule targeting VEGFR 1, 2, 3 PDGFRα and β, FGFR and c-kit. A phase III study, ICON6, evaluated its efficacy in recurrent platinum-sensitive EOC, comparing cediranib+ platinum-based therapy and cediranib in maintenance, to platinum alone and cediranib+ platinum. The increase in PFS and OS for the treatment with maintenance cediranib to chemotherapy alone was of 3.2 and 2.7 months respectively [198].

There are recent efforts in investigating immune based therapy in ovarian cancer. The new strategy based on blocking the complex PD-PD-L1 with Nivolumab has been tested in phase II clinical trials and produced an ORR of 15% in ovarian cancer [199]. At an interim analyses of phase Ib clinical trials for the PD-L1 inhibitor avelumab and the PD-1 inhibitor pembrolizumab, generated interesting results as well (ORR of 10.7% and 11.5% respectively) [200]. Another PD-L1 inhibitor, adalimumab, is currently in clinical trials in ovarian cancer.

Adoptive transfer of tumour-infiltrating lymphocytes, as completion to the chemotherapy in adjuvant setting [201], and treatment with CTLA-4 antibody in advanced ovarian cancer have also reached significant results [202].

Another approach combining immune and angiogenic targeting is the use of coledronate, a bisphosphonate, in a mouse model of ovarian cancer. Coledronate inhibits secretion of angiogenic cytokines both from endothelial cells and macrophages, resulting in a significant reduction of tumour size [203].

The wide range of current and experimental treatments targeting tumour stroma in ovarian cancer is reviewed in [204].

These new therapeutic approaches in ovarian cancer show that tumour microenvironment has now become an established target for the treatment of patients affected by this malignancy. Despite that, resistance remains a huge problem that makes urgent on one hand the search for new biomarkers that identify subgroups with different prognosis and sensitivity, and on the other hand the discovery of new targetable molecules.

3.4 STROMA-RELATED PREDICTIVE FACTORS

Regarding therapy outcome, FAP expression in tumour stroma was reported to be significantly associated with shorter time to recurrence after platinum therapy in patients with epithelial ovarian cancer [188]. Establishing new biomarkers aimed at predicting response to anti-angiogenic therapies is a challenge in ovarian cancer research. Bevacizumab has shown a limited gain in survival and predictive markers of response may achieve optimization. The search for biomarkers predictive of response to anti-angiogenic therapies involves different kinds of tumours and so far has not produced any clinically validated factor. Several attempts have been made with the measurement of baseline plasma VEGF levels, that seem to be higher in patients responding to bevacizumab in gastric cancer [205]; baseline or treatment induced changes in plasma levels of PLGF and VEGFR2 in renal cancer [206] [207], breast cancer [208] and lung cancer [209]. Contrasting results have been achieved with the use of functional imaging to measure heterogeneity of tumour vascular enhancement [210], and some encouraging data were collected when measuring circulating endothelial cells before and after antiangiogenic therapy in colorectal and breast cancer [211] [212].

Worthy to be mentioned is the evidence in favour of an association between VEGF genetic polymorphisms and differences in overall survival in breast cancer patients undergoing

combinatory therapy with paclitaxel and bevacizumab (in particular the genotype VEGF-2578 AA and the allele VEGF-1154 A with better prognosis) [213].

4. AIMS OF THE THESIS

The general aim of this thesis was to explore the composition of tumour microenvironment in serous ovarian cancer and identify its implications on survival and response to treatment.

The specific aims were:

1. To describe the biology of CAFs, vascular and perivascular cells and immune cells in the tumour microenvironment of serous ovarian cancer.
2. To investigate the prognostic and predictive potential of CAFs, vascular and perivascular cells and immune cells in the tumour microenvironment of serous ovarian cancer.
3. To compare the stromal, vascular and perivascular phenotype of serous ovarian cancer, to other selected tumour types

5. PATIENTS AND METHODS

5.1 PATIENTS AND TUMOUR MATERIAL

5.1.1 PAPER I-II AND III

In paper I, II and III the same cohort of ovarian cancer patients was investigated. The cohort was composed by women diagnosed with ovarian cancer from1986 to 2006 at the Department of Gynaecologic Oncology, University Medical Centre Groningen (Groningen, The Netherlands). Of the total 355 patients, tumour material was collected. 186 patients (52%) were chemo-naive and diagnosed with serous histologic type, and were included in our study (Figure 4). Clinico-pathological data were retrieved from medical records. Staging was performed according to FIGO. Classification and the three-tier differentiation grading were performed according to World Health Organization standards at the time.

For paper II and III only the subgroup of patients with tumor material derived from the primary ovarian site was considered for the analyses (N 138).

All patients gave informed consent. Studies were conducted in accordance with the Declaration of Helsinki principles and Institutional review board policies at University Medical Center Groningen. The study was also approved from the regional ethical committee of Stockholm (Dnr 2016/551-32).

Formalin-fixed and paraffin-embedded (FFPE) tissue blocks containing tumour in ovarian, omental and peripheral metastatic tissue and corresponding hematoxylin and eosin (H&E)-stained slides were retrieved from the pathology archives. Tumour specimens were obtained from the primary ovarian site in 138 patients, and matched tissues from metastatic lesions were also obtained from 91 patients. In 48 of 186 patients tumour tissue was obtained from only the metastatic site. A pathologist selected tissue microarray (TMA) cores as representative tumour areas and four 0.6 mm^2 core biopsies were taken from each tumour specimen and arrayed on a recipient paraffin block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). One to three tissue blocks per patient were available, taken from different tumour areas (primary site, omentum, peripheral metastasis). All arrayed samples were H&E stained to confirm the presence of tumour tissue.

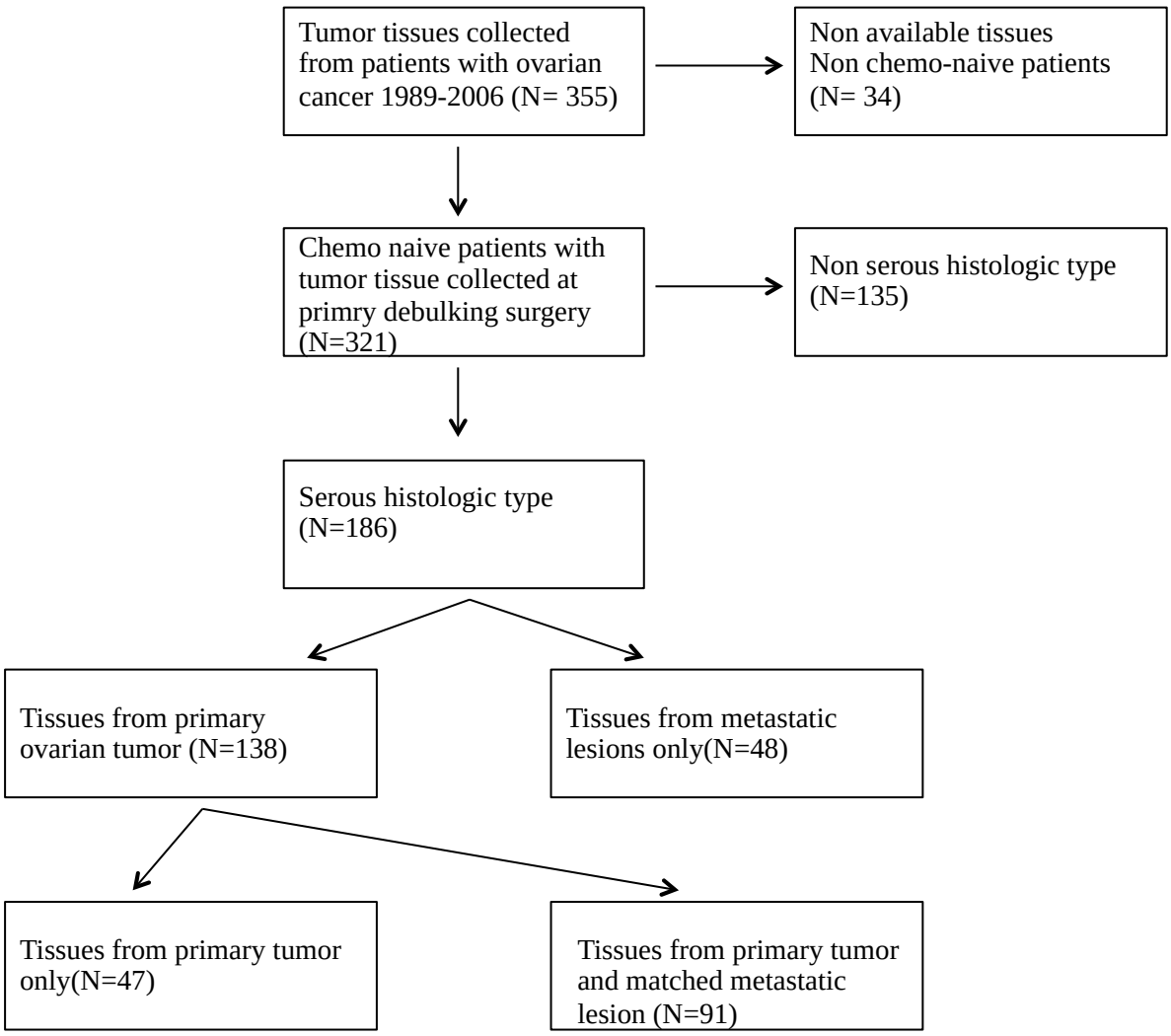

Figure 4: Consortium diagram of the study population for paper I, II and III.

5.1.2 PAPER IV AND V

For paper IV and V the same cohort of patients was investigated. All patients, diagnosed between 2002-2006 in Stockholm County with ovarian cancer, fallopian tube, primary peritoneal carcinoma and undesignated primary site according to the Swedish cancer registry, were screened for eligibility. Inclusion criteria were age above 18 years; high-grade serous histology; FIGO stage IIC to IV; no administration of chemotherapy prior to surgery or diagnostic biopsy, and availability of tissue from the pre-chemo tumour. Exclusion criteria were history of previous neoplastic disease (except for in situ cancer or basalioma); diagnosis at autopsy; prior chemotherapy; if either surgery or diagnostic biopsy was not performed (only cytology based diagnosis). All cases were re-classified by a pathologist (specialized in gynaecology) from the older three-tier differentiation grade to the new two-tier grade system, and only high-grade serous tumours were selected.

Of the 401 screened for eligibility, 199 patients met the including criteria, of which 32 did not have available tissue and 31 missed histology data verification or clinical data, thus 136 patients were included in the study (Figure 5). Only patients with available tissue from adnexal site were included in the analysis (N=116, Table 1) since stroma markers vary somewhat in different anatomical sites. The FIGO stage was classified according to the 1988 system [214]. Clinical data were retrieved from the charts, coded, and collected in case report files. Response was performed according to the Gynecological Cancer Intergroup (GICG) criteria [215]. The Regional Ethics Committee approved of the study (ethical permit number 2012/539-31/1).

FFPE biopsies and sections stained with hematoxylin and eosin were retrieved from tumour tissues obtained at primary surgery or diagnostic biopsies derived from chemo-naive patients. Sections were reviewed and a gynaecological pathologist confirmed diagnosis. Representative areas of the tumour were marked on the slide and from the corresponding blocks a punch of 1 mm diameter was cut and a TMA was built. Two punches per patients were taken, in line with the rules for TMA building at Karolinska University Hospital; if possible, one punch was retrieved from the primary site, and one from the metastatic omentum or peritoneum.

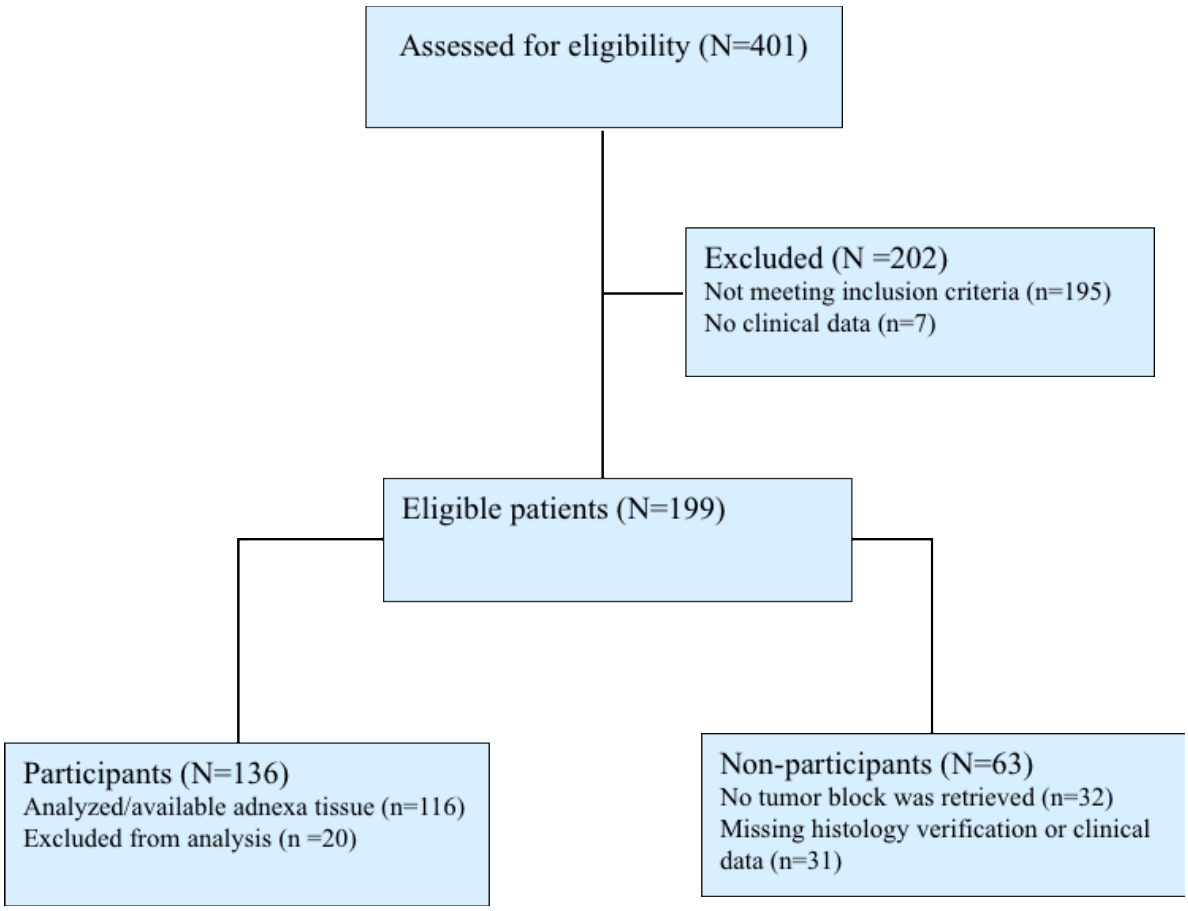

Figure 5: Consortium diagram for study population of paper IV and V.

5.2 METHODS: IMMUNOHISTOCHEMISTRY

5.2.1 PAPER I, II AND III

Primary antibodies used were recognizing α-SMA (anti human Smooth Muscle Actin, code M0851, Clone 1A4; Dako, Inc., Denmark (dilution 1:300)), PDGFRβ (PDGF Receptor beta 28E1 Rabbit mAb, 3169, Cell Signalling Technology, Danvers, MA (dilution 1:70)) and Desmin (Rabbit Anti-Human Desmin code HPA 018803-100UL Sigma Life Sciences, St Louis, MO (dilution 1:500)). Slides underwent treatment with secondary anti- mouse or anti-rabbit antibody (ImmPRESSTM-AP Polymer Anti-Mouse IgG, MP-5402 and ImmPRESSTM-AP Polymer Anti-Rabbit IgG MP-5401, Vector Laboratories, Burlingame, CA) and developed with Vector Blue AP substrate Kit (SK-5300, Vector Laboratories, Burlingame, CA). Sections were then incubated with primary antibody against CD34 M7165 Mouse mAb, DAKO, DAKO Agilent Technologies, Santa Clara, United States (dilution 1:100)) for 1 hour at room temperature, followed by ImmPRESS-AP Alkaline Phosphatase Polymer Anti-Mouse Kit at room temperature in a humidity chamber and

developed with Vector Red AP substrate Kit (SK-5100, Vector Laboratories, Burlingame, CA). (For details see [112] [113] and PAPER III in PAPERS section).

5.2.2 PAPER IV

Immunohistochemistry for FAP (rat antibody against human FAP 1:200, MABS1001, Vitatex, Stony Brook, NY) was performed following a VENTANA-ROCHE protocol and relative reagents. After deparaffinization slides underwent cell conditioning with Conditioner #1, Mild CC1, and Standard CC1. Then they were stained with primary and secondary antibodies, and treated with Ultramap anti Rt-HRP. After that tissues were counterstained with hematoxylin II and post counterstained with bluing reagent. An additional counterstaining with hematoxylin (Mayers HTX, 01820, Histolab, Sweden) was then performed manually.

Immunohistochemistry for CD8 (1:100, M7103, DAKO Agilent Technologies, Santa Clara CA) was performed following a VENTANA protocol and relative reagents. After deparaffinization slides underwent cell conditioning with Conditioner #1, Mild CC1, Standard CC1 and Extended CC1. Then primary antibody and OMap anti-Rb HRP were applied. Counterstaining with Hematoxilin II and post counterstaining with bluing reagent were done and an additional counterstaining with hematoxylin (Mayers HTX, 01820, Histolab, Sweden) was performed manually.

5.2.3 PAPER V

Single stainings for CD11c, CD80, CD163, FAP and CD8 were performed as follows: sections were deparaffinized, rehydrated and boiled at 110^{o} C for five minutes in deckloaking chamber with a pH 6 Buffer solution. Treatment in 3% H2O2 for 20 minutes was performed and after blocking with blocking solution (DAKO) slides were incubated with primary antibodies over-night at 4^{o} C. The antibodies used were 1:100 dilution of NCL-L-CD11c-563 Mouse IgG2a; Leica BioSystems (CD11c), 1:100 dilution of NCL-CD163 Mouse IgG1, Leica BioSystems (CD163) and 10 μg/ml of Monoclonal Mouse IgG1 Clone # 3771 MAB 140; Biotechne (CD80).

Sections were incubated with EnVision HRP anti-mouse kit (DAKO Agilent Technologies, Santa Clara, United States) for anti CD11c and anti Cd163 stainings, and with ImPRESS antimouse Ig Vector (MP 7402, VECTOR, Burlingame CA, 9410) washed and developed with DAB (3,3'-diaminobenzidine). A final counterstaining with Hematoxylin (Mayers HTX, 01820, Histolab, Sweden) was performed before mounting.

Procedures for staining of FAP (fibroblast activation protein) and CD8 have been described for paper IV.

Procedures for double staining with PDGFRβ -, desmin- or α-SMA-antibodies together with CD34 antibodies were similar to the ones described earlier for paper I, II and III [113] [112].

For double stainings with CD11c, CD80 or CD163 plus CD34 antibodies, the tissue was treated with the primary anti-macrophage antibodies described above, then washed and stained with VECTOR Blue Alkaline Phosphatase substrate Kit. Second treatment with anti-CD34 was performed as described earlier [113], liquid paramount RED from DAKO (K0640 DAKO Agilent Technologies, Santa Clara, United States) was used to develop CD34 staining.

Slides were subsequently scanned with Aperio Scanscope AT, and acquired through Image Scope for visualization.

Establishment work for the single and double staining procedures for CD11c, CD80, CD163 and CD68 were performed on some paraffin embedded whole section slides derived from a prospectively collected cohort of high-grade ovarian patients, approved by The Regional Ethics Committee of Karolinska Institute (ethical permit number 2012/596-31/4).

5.3 METHODS: DIGITAL AND MANUAL IMAGE ANALYSES

5.3.1 PAPER I

The double stained slides were scanned and, after quality selection, images were analysed using Image J software, with an algorithm developed in-house. CD34 staining was used to determine vessel density, mean vessel area and mean vessel perimeter. For perivascular-restricted measurements the areas surrounding the vasculature were analysed. Analyses of desmin-, PDGFRβ - and α-SMA- stained samples yielded information about average intensity of the staining with these three markers in the perivascular area (perivascular intensity). To obtain values for perivascular fraction, individual vessels were classified as ‘uncovered’ or ‘covered’ and ratio of covered vessels over total vessels per case was thereafter calculated, to yield the perivascular fraction metric. PDGFRβ - and α-SMA-staining were also used to determine the stroma fraction, defined as the fraction of total tumor area positive for these markers. The stroma fraction was calculated as the sum of all positive regions divided by the total tumor area. In the case of PDGFRβ analyses this step also included exclusion of 35 cores with positive epithelial staining. Finally, PDGFRβ and

α-SMA-staining were used to obtain values for PDGFRβ - and α-SMA - intensity (stroma intensity) by calculating the average intensity of PDGFRβ - and α-SMA-staining in the marker- positive area.

Together these analyses, performed on the CD34/ α-SMA, CD34/desmin and CD34/PDGFRβ -staining yielded quantitative data for 13 different stroma-related metrics. For details see [113].

5.3.2 PAPER II

The scanned double stained slides used for study I underwent, after quality selection, image analysis using Image J software, with an algorithm developed in-house (R 3.2.2 GUI 1.66 Mavericks build (6996) http://www.R-project.org).

CD34 staining was used to determine vessel density, median vessel diameter and median vessel area. Perivascular intensity (PVI) was defined as median optical density (OD) of the perivascular staining.

To measure the heterogeneity of PDGFRβ within each tumor, the inter-quartile range of all vessels in each tumor was calculated (IQR). For detail see [112].

5.3.3 PAPER III

The scanned double stained slides used for paper I underwent, after quality selection, image analysis using Image J software [112].

Vascular and perivascular stainings were evaluated as described above and in [112].

Marker-positive stroma area was defined as the area of the analysed sample, having expression of the marker over a pre-defined baseline level; the perivascular regions (defined as above) and vessel regions were excluded from the marker-positive stroma area. Marker-positive stroma fraction was defined as ratio between marker-positive area and total analysed sample area. Marker-positive stroma intensity was defined as median of the pixels intensity of marker staining inside marker-positive stroma area.

The list of such metrics for every individual vessel in each sample was collected. A case-based median of these values was calculated. To quantitate the heterogeneity of the distribution of the vessels in the sample, the difference between 1^{st} and 3^{rd} quartile was calculated in each case - inter-quartile range (IQR), yielding case-based value for the metric "vessel distance IQR".

5.3.4 PAPER IV

For FAP scoring, slides were reviewed separately by a pathologist and an oncologist. Only cases with availability of tumour material from adnexal site were considered for the analyses (all cases of undesignated site and primary peritoneal cancer were spread to the adnexa). FAP fraction positive stroma on total stroma and FAP intensity of positive stained stroma, were scored independently on a semi quantitative scale and a consensus was found between the two observers. FAP fraction was scored on a 5 points scale (0: 0% of stroma area stained by FAP, 1: 1-10%, 2: 11-50%, 3: 51-95%, 4: 96-100%) and FAP intensity was scored on an optical 4 points scale (0 to 3); descriptive images are provided in Figure 2. Two metrics were then produced: FAP positive fraction in the primary tissue and FAP intensity in the primary tissue. For the survival analyses FAP positive stroma intensity was dichotomized in low (score 0 and 1) and high (score 2 and 3).

CD8 density was scored by an oncologist on a semi-quantitative five points scale (0: 0% of stroma area covered by CD8 positive cells, 1: 1-10%, 2: 11-50%, 3: 51-95%, 4: 96-100%), as CD8 density in the epithelial areas of the primary tissue and CD8 density in the stromal areas of the primary tissue (Fig. 3). Grade 4 was never reached in stromal CD8 density scoring, so for survival analyses CD8 stromal density was used as dichotomized in low (score 0 and 1) and high (score 2 and 3). A similar scoring procedure is used by Donnen et al [216]; in our analysis, high density of CD8 scoring coincides with theirs, while our definition of low density corresponds to low and intermediate in their cohort.

5.3.5 PAPER V

For the macrophage single stainings markers density was scored on a semi-quantitative five-point density scale (0: absent staining; 1: 1-10% positivity, 2: 11-50% positivity, 3: 51-95% positivity; 4: 96-100% positivity) separately in histo-morphologically defined tumour epithelial and tumour stromal areas.

Procedures for staining and scoring of FAP (fibroblast activation protein) and CD8 have been described earlier for paper IV. Algorithms developed in-house were used for double stainings of PDGFRβ, desmin and α-SMA plus CD34 as described in paper II and III.

For the macrophage double-stainings images were analysed with Image J software. Digital-image analyses of the double staining with either CD11c, CD80 or 163 antibodies together with CD34 antibodies was used to collect three "metrics" for each of the macrophage-related markers. *"Total tumour density"* was determined by calculating the fraction of total "region

of interest" that was positive for marker. Two metrics were collected related to marker density in perivascular areas; "*perivascular area 1 density*" and "*perivascular area 2 density*" (see also schematic Supp. Fig. 1). Perivascular area 1 was defined as the area most close to CD34 positive regions extending 30 pixels away from the CD34 positive area. Perivascular area 2 was defined as the area surrounding perivascular area 1 and extending 30 pixels peripheral of perivascular area 1 (two pixels corresponding to one micrometre). "*Perivascular area 1 density*" and "*perivascular area 2 density*" were determined by calculating, for each case, the fraction of marker-positive area in all summed-up perivascular areas 1 and 2, respectively. Marker positive area is defined as the area where the intensity of the marker is above a threshold defined at a preliminary visual evaluation.

Validity of the automated scoring was tested by comparing correlations between manually scored and digitally scored perivascular density area 1 and total density from 30 selected cases of CD80/CD34 double-stainings. As shown in Supp. Table 1, correlation coefficients were overall high with Goodman and Kruskal´s gamma index values of 0.9 and 0.8 respectively.

5.4 METHODS: STATISTICAL ANALYSES

5.4.1 PAPER I AND II

The Spearman two-tailed test was used for correlation estimation between stromal markers expression, a correlation coefficient of 0.5 and a *p*- value <0.01 were used as reference threshold values. Cox proportional hazards model and the Kaplan-Meier estimator were used to analyse the association between the markers and overall survival (OS). Kaplan-Meier survival analysis was used to analyse survival rates and a multivariate Cox regression model was used to calculate hazard ratios of the clinical-pathological factors and the stroma related metrics for patients' survival and to determine their independence. The survival findings were confirmed by backward selection. Associations between stroma metrics and clinico-pathological characteristics of the patients were performed with Chi-square test. All tests were done at the 95% significance level and were performed using SPSS version 22 (SPSS Inc., Chicago, IL). Forest Plot was done using R 3.2.2. Meta package.

5.4.2 PAPER III

For comparison between tumour types for marker-intensity-related metrics, original data for each tumour type was normalized and given values between 0 and 1. Normalization was done case-wise for stroma intensity and vessel-wise for perivascular status. Normalization

addressed the issue of skewed distribution of data by skewness adjustment as described by Vanderviere [217] and vessel distance IQR" metrics were dichotomized per the median.

All tests were done at the 95% statistical significance level and were performed using SPSS version 22 and 23 (SPSS Inc., Chicago, IL) and Rstudio (Version 0.99.489 – © 2009-2015 RStudio, Inc.). Differences between tumour types regarding case-based values for stroma metrics, were determined using Mann-Whitney U test. Correlations between case-based stroma metrics, as analysed in Fig. 2, were determined by Spearman correlation test for the pair-wise analyses. Log Rank Tests and Cox Regression Models were used to estimate relationships between analysed metrics and overall survival. Associations with the clinico-pathological characteristics were evaluated with Pearson´s Chi Square test.

5.4.3 PAPER IV AND V

Spearman correlation test and Goodman and Kruskal's Gamma correlation test determined correlations between case-based stroma metrics. Comparisons of means were performed through Wilcoxon test, Pearson Chi-square test and Mann-Whitney test.

Overall survival (OS) was defined as survival from date of diagnosis to date of death of any cause. Progression- free survival (PFS) was defined as the time frame from the date of diagnosis to progression, recurrence or death from any cause (whichever came first). Objective response rate (ORR) was defined as the proportion of patients with a response at the end of treatment (EOT): a partial regression or complete regression (PR and CR) was considered response, while stable disease and progression of disease (SD and PD) were considered as absence of response. Significant differences in OS and in PFS were estimated using Log Rank tests and Cox Regression proportional hazard models. All variables showing a significant p value (< 0.05) at the univariate analysis were entered into the multivariate model. FIGO stage, age at diagnosis and residual tumour after primary surgery (if applicable) were the clinical variables included in the multivariate Cox regression model. Correlation analyses were performed through Pearson Chi-square test. All statistics were performed in SPSS, version 23.0.

6. RESULTS AND DISCUSSION

6.1 VASCULAR AND PERIVASCULAR CHARACTERISTICS

6.1.1 VASCULAR AND PERIVASCULAR BIOLOGY IN SEROUS OVARIAN CANCER

In the first two studies (PAPER I and II) we analysed perivascular and vascular features of serous ovarian cancer, and investigated the presence of prognostic markers related to tumour microenvironment.

A notable observation from the investigation of vessel features (as vessel density, lumen area and lumen perimeter) and perivascular features (as coverage with desmin, α-SMA and PDGFRβ), was the total absence of correlations between these two groups of features. This might suggest an independent regulation, during vessel maturation, of features strictly related to endothelial cells from the ones related to perivascular cells. A significant correlation lacked also among the three subtypes of pericytes, suggesting again the existence of different regulatory circuits controlling subtypes of pericytes with different functions. When comparing these two entities in primary ovarian tissues and matched omentum metastases, we found, again, no significant correlation, with the exception of PDGFRβ perivascular intensity. This intra-patient heterogeneity may be important to be taken into account when considering anti-angiogenic approaches in patients with advanced and metastatic tumours.

6.1.2 PROGNOSTIC SIGNIFICANCE

When analysing the potential impact of perivascular metrics on survival of serous ovarian cancer we found that high PDGFRβ positive perivascular intensity (PAPER I) and high heterogeneity of PDGFRβ perivascular intensity, defined by the interquartile range of the marker-intensity (PAPER II), were related to worse prognosis (figure 6B and figure 7).

Recent evidences reporting an interaction between pericytes and macrophages in promoting metastatization through a PDGF-BB related pathway [107] support our finding of a particular subset of perivascular cells impacting on survival.

In ovarian cancer, characterization of perivascular cells may have a double role: on one hand it can be used as a way to stratify patients undergoing anti-angiogenic therapies [218] on the other it can help to improve these treatments implementing them with a pericytes-targeted

approach. Dual targeting with VEGF and PDGF blockade has indeed generated interesting results in vitro and in vivo in this malignancy [219] [220].

According to our data, heterogeneity of perivascular coverage seems to impact negatively on prognosis (PAPER II). It is known that tumour cells intra-patient heterogeneity is a driver for adverse clinical outcome [221], our study, though, points out at the perivascular heterogeneity as a marker or driver for cancer aggressivity. Perivascular heterogeneity can indicate the presence of vessels in different stages of maturation, which might cause non-homogeneous tumor vascularization and oxygenation, fuelling in tumor cells the shift towards more aggressive phenotypes [95]. This process makes "vessel normalization" a desirable target of cancer therapy. As suggested by our findings, in serous ovarian cancer, vessel normalization needs to be achieved implementing control of perivascular cells together with endothelial cells. Therefore, the results of our first two articles prompt new studies supporting the role of perivascular cells and PDGFRβ, as prognostic factors and molecular targets for ovarian cancer therapy.

Notably, neither vessel density nor vessel diameter, were related with overall survival in this cohort of patients.

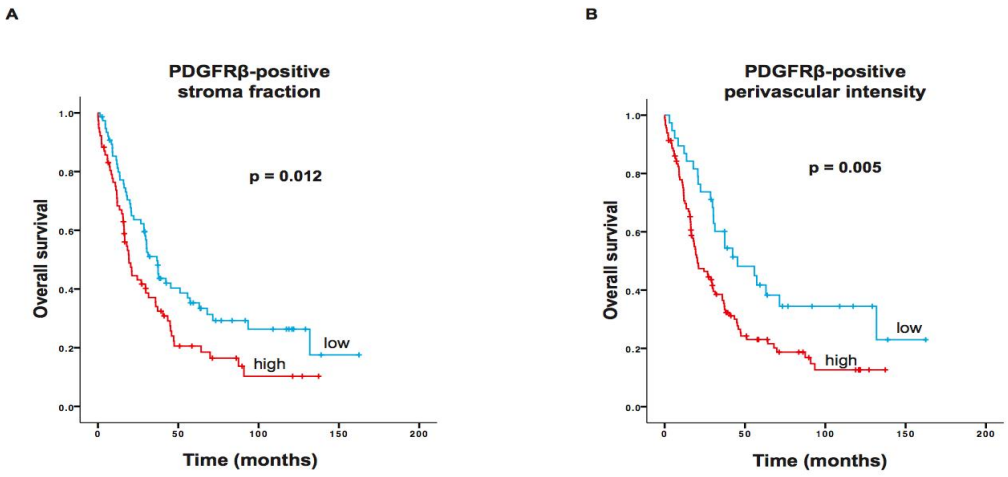

Figure 6 A: Survival curves for high and low PDGFβR positive stroma fraction.

Kaplan-Meier graph shows worse overall survival for high PDGF βR positive stroma fraction as compared to low PDGFβR positive stroma fraction in serous ovarian cancer (n=186 patients) (p= 0.01 2, Log Rank).

Figure 6 B: Survival curves for high and low PDGFβR positive perivascular intensity. Kaplan-Meier graph shows worse overall survival for high PDGF βR positive perivascular intensity as compared to low PDGF βR positive perivascular intensity, in 186 patients (p= 0.005, Log Rank).

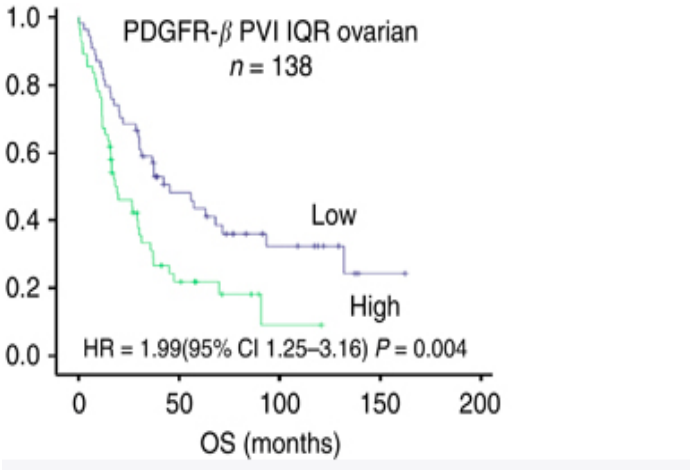

Figure 7: Survival curves for high and low PDGFRβ positive perivascular intensity IQR. Kaplan-Meier graph shows worse overall survival for high PDGFRβ positive perivascular intensity IQR as compared to low PDGFRβ positive perivascular intensity IQR, in 138 patients (p= 0.004, Log Rank).

6.2 CAFs

6.2.1 CAFs IN SEROUS OVARIAN CANCER

In PAPER I and IV, we describe different subtypes of cancer-associated fibroblasts, composing the stroma, of serous ovarian cancer. In PAPER I we analyse two subtypes, the α-SMA positive ones and the PDGFRβ positive ones; in PAPER IV we focus instead on FAP+ fibroblasts.

In the first study, we notice an independence of the two cell subsets from each other, confirming once again the existence of different classes of tumour-associated stroma cells, previously reported by others [73] [222]. We also noticed a significant correlation between perivascular cells and fibroblasts both positive to PDGFRβ or α-SMA. These data suggest a shared origin for perivascular cells and stroma fibroblasts. Some lineage tracing experiments on fibrosis and brain scarring, have indeed implied a perivascular cell of origin for interstitial fibroblasts and glial cells [223] [170]. When we compared primary sites and matched metastatic sites, we found a discordance of most of the stroma parameters, as we previously reported about vascular and perivascular features. This finding is in line with what has previously been reported for breast and colon cancer [224].

A third CAFs subtype is described in PAPER IV, the FAP+ subtype. The FAP protein seems to be exclusively expressed by fibroblasts (differently than PDGFRβ which might be

found in tumour cells as well) with a high inter-case variability, regarding both intensity and percentage positive of the stroma area.

6.2.2 PROGNOSTIC SIGNIFICANCE

In PAPER I we investigated the prognostic impact of the stroma-related metrics and revealed the presence of a significantly poor survival associated to high PDGFRβ stroma fraction, both in uni- and multi-variate analyses (figure 6A). Data on the negative impact of PDGFRβ positive stroma on survival, has previously been reported for breast and prostate cancer [72] [69]. Our group has recently shown with in vitro and in vivo studies, an increase in invasiveness and metastatization of colorectal cancer cells when cultured with PDGFRβ-activated fibroblasts [225].

FAP expressing cells instead, as analysed in PAPER I, did not show any impact on prognosis in the cohort of high-grade serous patients.

6.2.3 PREDICTIVE SIGNIFICANCE

The patient cohort available for PAPER I did not allow us to analyse the predictive value of the examined markers, due to lack of specific data on treatment. However, in PAPER IV we had a cohort of high-grade serous ovarian cancer patients where detailed clinical and treatment data was collected. Therefore, we could explore the potential impact of FAP+ CAFs on response to platinum-based treatments. We found on the cohort of patients with high density of stromal CD8+ cells and high intensity of FAP+ stroma cells to be associated with early relapse (<6 months after last platinum treatment, platinum-resistant) compared to low intensity of FAP ($p=0.04$). In the "high-density" CD8 group had also a shorter progression-free survival (PFS) in patients with high intensity FAP positive stroma, as compared to the group with low intensity FAP+ stroma (figure 8).

Several in vivo and in vitro tumour biology studies have supported the existence of an immune-modulating effect for FAP+ fibroblasts [55] [139]; both these studies show that FAP+ CAFs interfere with the immune control of tumours, mostly interacting with T-lymphocytes. In our analyses the modulatory effect of FAP+ cells, on sensitivity to therapy is detectable when there is a high immune control of the disease, expressed by density of T+ cells.

Immunogenic cell death is a mechanism involved in the tumoricidal effect of platinum compounds, and it is triggered by different biological events sequential to the activity of the

chemotherapeutic agents on the tumoral cells [226] [227]. Our work suggests that immunogenic cell death, as a result of carboplatin treatment of high-grade serous ovarian cancer, undergoes modulating by of FAP+ fibroblasts.

Clinical trial on immune-targeting in ovarian cancer, supported by the evidence that infiltrating T cells confer a good prognosis [124], are ongoing with alternating results [199]. Inhibition or depletion of FAP+ fibroblasts, may improve the outcome, conferring an advantage in cases in which FAP positive stroma cells weaken immune activation. Although the results need indeed to be validated in an independent cohort, FAP may be a targetable molecule to enhance sensitivity to therapy in patients with a strong CD8 immune response.

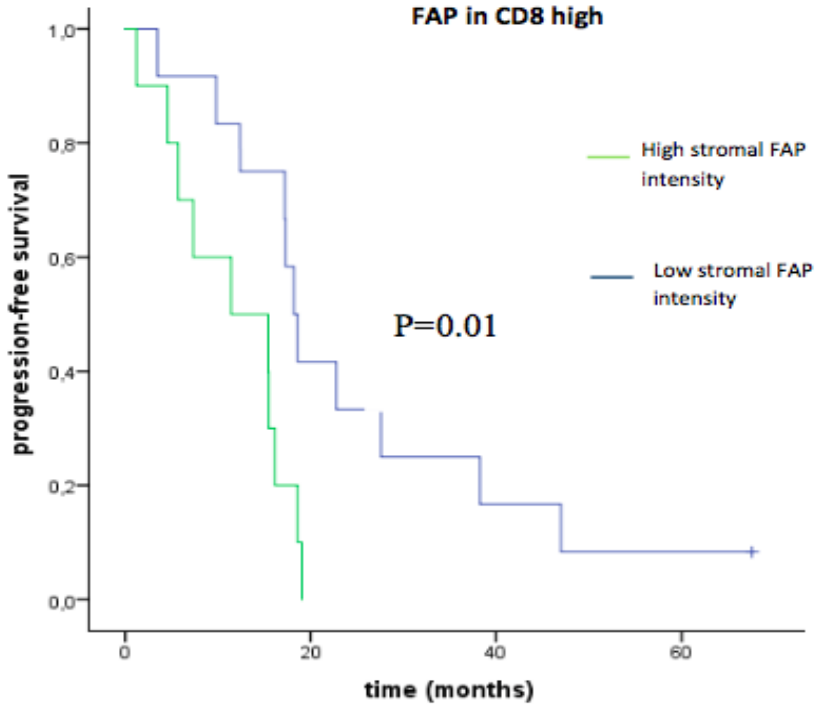

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Figure 8: Progression-free survival curves for high and low stromal FAP intensity among patients with measurable diseases at start of platinum-based chemotherapy in high-grade serous ovarian cancer (N=73).

Log-rank test (p 0.01) showed a shorter PFS in patients with FAP high intensity as compared to FAP low intensity in the sub-population of patients with high CD8 stromal density

6.3 MACROPHAGES

6.3.1 MACROPHAGES DISTRIBUTION IN HGSOC, AND INTERACTIONS WITH TME

Functionally distinct macrophage subsets exist in the tumour microenvironment and are characterized by specific markers expression and compartment-related regulatory functions. In PAPER V we investigated this subject, by profiling CD68 positive subtypes of cells, according to their expression of CD11c, CD80 and CD163 respectively; density was evaluated in the total tumor and in four different tumour compartments in a clinically well-annotated cohort of high-grade serous ovarian cancer.

CD163-positve cells were the most abundant among the marker-defined cell subsets. CD11c and CD163 subsets were strongly correlated to each other in tumour stroma and tumour epithelial areas, whereas CD80/CD163 in tumour epithelial areas and CD11c/CD80 in the perivascular areas showed the lowest correlations.

When associations with other stroma features were explored, a negative correlation was detected between perivascular desmin-expression and perivascular CD163 cells, suggesting a displacement operated by this macrophage subtype, usually recognized as “M2”, towards pericytes in the vessel walls. A role of M2 macrophages in regulation of permeability enacted through physical contact between macrophages and endothelial wall has been recently described [228]. Positive significant correlations were observed between FAP+ fibroblasts and density of stromal CD11c and CD163+ cells, while absent or very low correlation was found with the other subtypes of stroma fibroblasts, again underlining the immune-related role of FAP+ cells. Significant positive associations were also revealed between stromal density of the CD8+ T cells and stromal density of CD11c, Cd80 and Cd163 positive cell subsets.

6.3.2 PROGNOSTIC SIGNIFICANCE

Despite the solid evidence that HGSOC maturates and disseminates in a stroma-rich environment highly populated by macrophages, the prognostic implications of these cell types have been poorly explored. Our analyses of overall survival identified that patients with high stromal CD11c density had a better prognosis as compared to patients with low CD11c stromal density (figure 9).

The association of CD11c positive cells with reduced tumour aggressivity, which translates into a better survival, might be explained considering their active bidirectional communication with Th1 cells [229]. Nonetheless the stromal restriction of the signal suggests the possibility of an interaction between CD11c-postive cells and CAFs, which are the dominating cell type of the ovarian tumour stroma. Findings from recent years have postulated the existence of functionally distinct CAF subsets with anti- or pro-tumoral effects [230] [231] [232] [233]. Some studies have also reported functionally relevant interactions between macrophages and CAFs [234, 235]. The possibilities that CD11c-positive macrophages exert inhibitory effects on pro-tumoral CAFs, or stimulate anti-tumoral CAF subsets, should therefore be further explored.

6.3.3 PREDICTIVE SIGNIFICANCE

When investigating relationships between markers and response to treatment we identified a possible association between perivascular CD80-positive cells and better response to platinum treatment (figure 10), an effect that seemed even clearer in patients with measurable disease at start of primary platinum-based treatment (PAPER V).

CD80 is a co-stimulatory molecule, whose expression is necessary for the activation of Th1 cells and it´s triggered by inflammatory stimuli [236]. The particular association with response to treatment suggests possible effects of CD80-positive cells on drug delivery. Moreover, we found no correlation between CD80-positive expression and vessel density, indicating effects on quality and maturation rather than quantity of vessels. Vessel-normalizing functions of macrophages have indeed been suggested by previous studies. Wenes et al. recently demonstrated that macrophages could promote the formation of stable and functional vessels, though mechanisms involving activation of mTOR [237]. Furthermore, macrophage-induced normalization of tumour vasculature has also been shown to occur through down-regulation of PlGF [238].

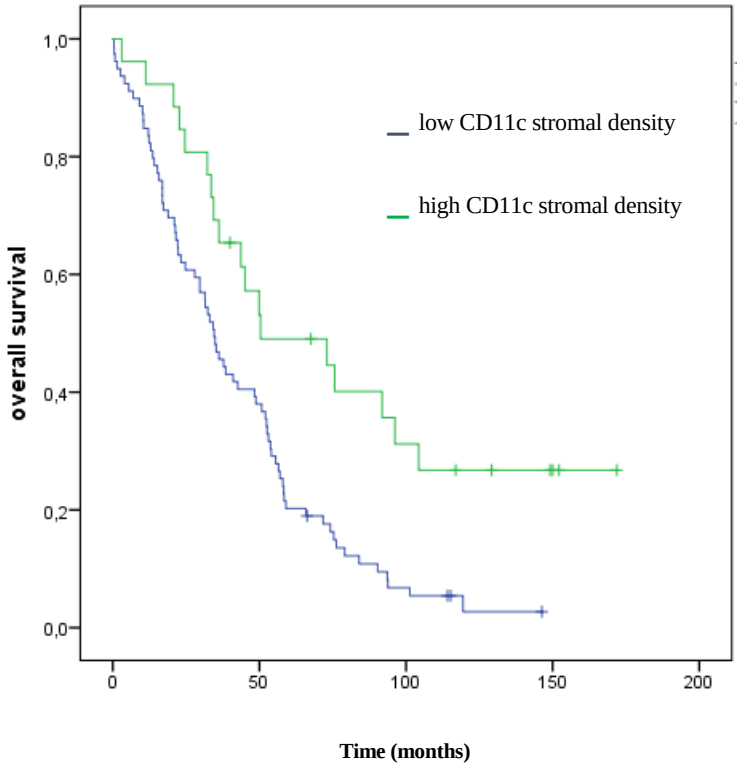

Figure 9: Overall survival according to CD11c tumor stromal density among patients operated for high-grade serous ovarian cancer.

Log-rank test (p-value) showed that patients with high CD11c stromal density had a longer OS as compared patients with low CD11c stromal density**.**

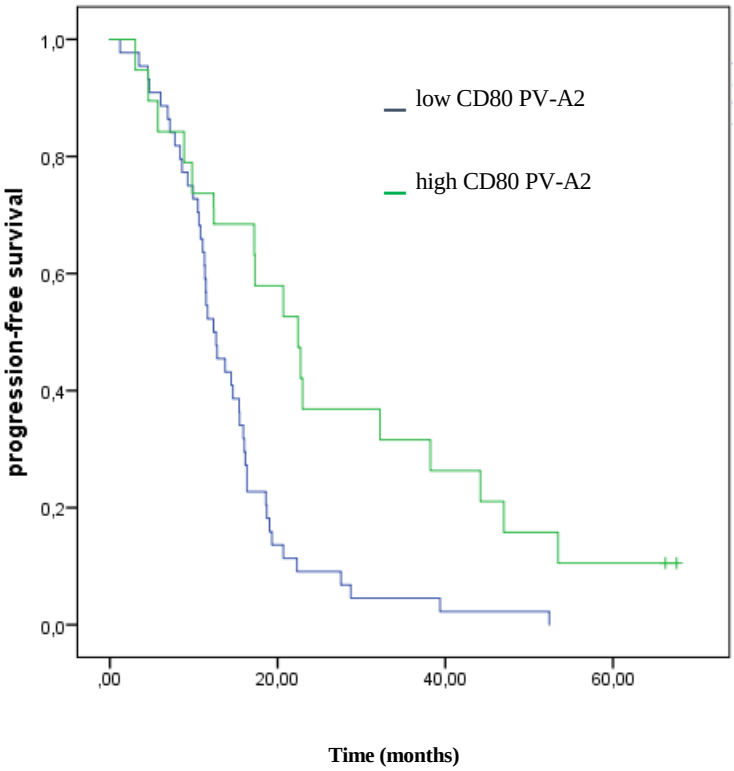

Figure 10: Progression-free survival (PFS) among patients with measurable diseases at start of platinum-based chemotherapy in high-grade serous ovarian cancer according to fraction perivascular (PV) CD80 positive cells in the A2 area (A2%).

Log-rank test (p-value) showed that patients with high CD80 fraction of cells in the A2 area had a longer PFS as compared patients with low CD80 fraction of cells in the A2 area.

6.4 COMPARATIVE ANALYSES OF TME IN OVARIAN, RENAL AND COLORECTAL CANCERS

6.4.1 SIMILARITIES AND DIFFERENCES

In paper III we aimed at studying the composition of tumor microenvironment in serous ovarian cancer, as compared to renal cell and colorectal cancer, through the investigation of several vascular, stroma and perivascular features, mostly related to PDGFRβ.

Firstly, we investigated if the three tumor types demonstrate significant differences regarding features such as vessel diameter, vessel density and distribution, perivascular and stromal PDGFRβ expression. Secondly, analyses of the cohorts were used to explore the prognostic significance of a novel vascular metric, "vessel distance inter-quartile range (IQR)", describing the intra-case heterogeneity regarding vessel distribution.

The comparisons between the three tumor types demonstrated a set of differences. Vessel density of renal cell cancer was significantly higher than in colorectal and ovarian cancer. Vessel size was highest in ovarian cancer, while PDGFRβ positive stroma abundance was higher in colorectal cancer. Concerning perivascular status, colorectal cancer displayed higher levels of perivascular PDGFRβ expression than the other two tumor types. Intra-case heterogeneity of perivascular PDGFRβ expression was also highest in colorectal cancer.

High "vessel distance IQR" was significantly associated with poor survival in both renal cell cancer and colorectal cancer (figure 11).

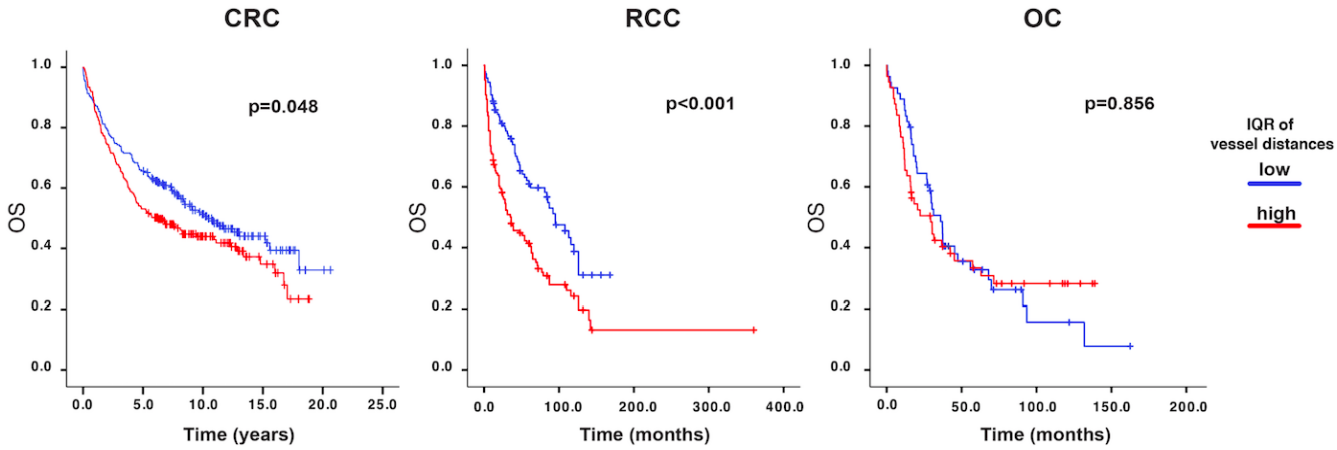

Figure 11: Survival curves for high and low IQR of vessel distances in CRC, RCC and OC. Kaplan-Meier graph shows worse overall survival for high IQR of vessel distances in RCC and CRC, as compared to low IQR of vessel distances.

6.4.2 DEFINITION OF DIFFERENT "PHENOTYPES"

This study suggests that ovarian, renal and colorectal cancer display specific "vascular/perivascular" and "tumor/vessel"-"stroma/vessel" configurations, potentially related to their sensitivity to anti-angiogenic therapies.

Renal cell cancer shows a phenotype with high vascularization and low perivascular coverage, possibly related to a VEGF-dependent and endothelial cells-dependent angiogenic program, as sustained by previous evidences [82, 239].

Studies by Smith et al [240] identified two tumor vascular phenotypes referred a “tumor-vessel” phenotype with vessels distributed predominantly in tumor cells-rich areas and a “stroma-vessel” phenotype with vessels in stroma fibroblasts rich-areas. Based on analyses of experimental cancer models, the study concluded that these two vessel phenotypes were linked to anti-VEGF sensitivity such that the “tumor vessel”-phenotype was more sensitive. Our analyses of stroma phenotypes of the three tumor types, and their known sensitivity to anti-VEGF-treatment are compatible with the concept proposed by Smith et al.

Renal cell cancer displays a “tumor-vessel” phenotype and also high sensitivity to anti-angiogenic drugs like bevacizumab, sunitinib and pazopanib [241] [242]. Ovarian cancer is characterized by a “tumor/vessel” configuration but also by moderate/high vessel maturation. This combination might underlie its partial sensitivity to anti-angiogenic therapy, mostly in combination with chemotherapy, but also as maintenance in mono-treatment, [33, 34]. Colorectal cancer instead has a phenotype with fewer and highly covered vessels immersed in a rich stroma (“stroma/vessel” phenotype), which could be the cause of the lower sensitivity to mono-treatment with antiangiogenic drugs [243, 244].

The phenotype patterns identified in these analyses require further validation. Their functional associations should also be experimentally tested. Potentially they can be used as signature to identify cases in ovarian or colorectal cancer associated with higher sensitivity to anti-angiogenic therapies. Furthermore, it could be tested whether the anti-VEGF-benefit in ovarian cancer is particularly strong in cases displaying the most typical “perivascular-low” phenotype.

7. LIMITATIONS

Throughout the different studies and in all the five papers we used two tumour collections both arranged as tumour microarrays (TMA). Despite the several advantages that this method has, such as availability of a large number of tissues with the smallest amount of resources needed to store and analyse them, plus the reduction in inter-cases analyses differences thanks to the arraying feature, the availability of a small area of tumour and of a restricted number of punches can sometimes limit the possibilities of catching tumour related heterogeneity.

In paper I, II and III we had access to a TMA consisting of up to four cores per tissue, but in study IV and V due to regulations of the pathology department we could get just one punch per tissue block.

Therefore, we performed a quality analysis to check if the afore mentioned four cores were concordant for FAP scoring, allowing us to draw proper conclusions using our TMA with just one punch per patient. Staining and scoring techniques of the "four cores-TMA" were the same as for the "one core-TMA". Just in ten out of 40 cases, one core of the four stained, was discordantly scored.

For four of the five studies, we extensively used digital image analysis, which is often questioned for its adherence to visual scoring. We addressed this issue in paper V, where we showed that concordance between manual and digital scoring for 30 cases of the cohort used in the same paper, stained with CD80/CD34. The correlations between manual and digital scoring were quite high, with coefficients of 0.8 for the scoring of total area and of 0.9 for the scoring perivascular area (Kruskall and Goodman Gamma test).

Regarding the patient selection, in paper I, II and III we used a cohort composed of cases of serous ovarian cancer not subjected to the two-tier grading system that now is used to grade ovarian cancer. Furthermore, patients were included starting from thirty years ago, which translates into a significantly different therapeutic approach, for example the surgical one. Despite that, for the same reason, we could benefit from a long follow up time, which was strength-point in our survival analyses.

The biggest limitation of our five studies is represented by the lack of functional investigations. Nonetheless our evidences generated several mechanistic speculations that could lead in vitro and in vivo experiments.

Despite the presence of some limitations that we tried to overcome, our studies could benefit of well-selected tumor material, detailed annotated clinical information and pathology revision (for IV and V), and deeply established laboratory techniques.

8. CONCLUSIONS AND FUTURE PERSPECTIVES

In our five studies, we characterized the tumour microenvironment of serous ovarian cancer, with the aim of identifying tumor-type-specific properties and to identify factors impacting on prognosis and sensitivity to therapy.

We described serous ovarian cancer according to quality and quantity of its vasculature, perivascular coverage, composition in stroma fibroblasts, and in macrophages, and learnt that these features display different degrees of interdependence.

We investigated the prognostic and predictive impact of the afore mentioned cell types in the tumour microenvironment and detected significant association of specific stroma, perivascular and macrophage markers with overall survival and of different stroma and macrophage markers on response to platinum-based therapy.

Moreover, we described a range of phenotypes related to vascular, perivascular and stroma fibroblasts compositions of tumours, which might reflect the wide spectrum of sensitivity to anti-angiogenic therapy of different tumour types. Among these phenotypes, we identified serous ovarian cancer as belonging to a "tumour/vessel" and "perivascular- dependent" phenotype, compatible with an intermediate level of sensitivity to vessel-targeted drugs.

We summarize our findings in a generic picture describing an "aggressive" type of serous ovarian cancer on one side, characterized by a bad prognosis, and a "resistant" type of serous ovarian cancer, characterized by the lowest sensitivity to therapy (figure 12).

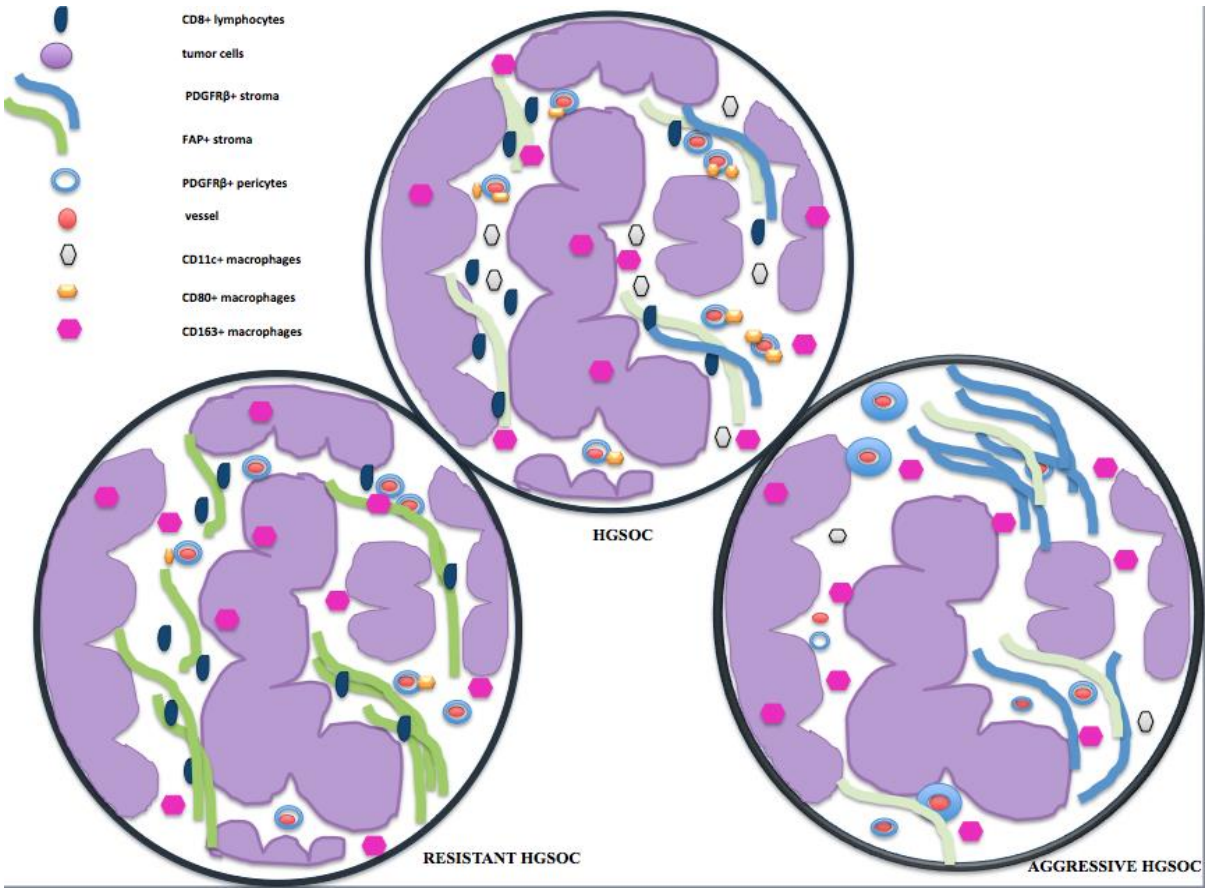

Figure 12: Schematic representation of the "aggressive" HGSOC with poor prognosis and the "resistant HGSOC" with low sensitivity to platinum-based therapy. The aggressive phenotype has high PDGFRß positive stroma fraction, high intensity of PDGFRß positive perivascular intensity, high heterogeneity of PDGFRß positive perivascular intensity and low density of CD11c positive macrophages. The resistant phenotype has high FAP+ stroma intensity, high CD8+ lymphocytes density, low CD80+ macrophages density.

In the future, we aim at investigating the prognostic impact of the parameters analysed in paper I, II and III for serous ovarian cancer, in the more recent and better characterized cohort of high-grade serous ovarian cancer patients collected for study IV and V. We also aim to validate our candidate predictive markers from paper IV and V in an independent cohort of high-grade serous ovarian cancer patients and to explore them with regard to mechanism through experimental studies.

Finally we would like to explore with different techniques such as immunofluorescence, confocal imaging and single-cell sequencing, the heterogeneity of stroma-derived cells in high-grade serous ovarian cancer.

Our data support future studies on the prognostic and predictive relevance of the different cells in the tumor microenvironment of ovarian cancer.

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